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Subpopulation Status of Lake Whitefish (*Coregonus clupeaformis*) in Lake Winnipeg

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Kristofferson, A. H., and J. W. Clayton. 1990. Subpopulation status of lake whitefish (*Coregonus clupeaformis*) in Lake Winnipeg. Can. J. Fish. Aquat. Sci. 47: 1484–1494.

Spawning lake whitefish in Lake Winnipeg from Dauphin River–Lake St. Martin, Traverse Bay, Berens River–Poplar River–Big Black River and Grand Rapids and in Little Playgreen Lake are accorded subpopulation status based on morphometric measurements, meristic counts, and differences in frequencies of alleles at the muscle glycerol-3-phosphate dehydrogenase (*g-3-pdh-b*) locus. The frequency of the *g-3-pdh-b-3* allele in samples from Dauphin River–Lake St. Martin was 0.77 compared with 0.46 for whitefish from the remainder of Lake Winnipeg and Little Playgreen Lake. Two loci for isocitrate dehydrogenase and one lactate dehydrogenase locus are also polymorphic in these fish but no significant allele frequency differences were found at these loci among the samples of spawners compared above. Hatchery-reared lake whitefish, obtained from stock in Clearwater Lake and William Lake and planted as fry or eyed eggs in Lake Winnipeg at Dauphin River and Grand Rapids, do not appear to have made a detectable genetic contribution to the local whitefish stocks, based on differences in *g-3-pdh-b* and *idh Bβ* allele frequencies among parent stock and spawners captured near the release sites.

Les grands corégones géniteurs de la rivière Dauphin, du lac St-Martin, de la baie Traverse, de la rivière Berens, de la rivière au Tremble, de la rivière Black, des rapides Grand et du petit lac Playgreen, se déversant tous dans le lac Winnipeg, sont considérés comme une sous-population d'après des caractéristiques morphométriques et méristiques et des différences pour ce qui est des fréquences des allèles au locus musculaire glycérol-3-phosphate-déshydrogénase (*g-3-pdh-b*). La fréquence de l'allèle *g-3-pdh-b-3* dans les échantillons recueillis dans la rivière Dauphin et le lac St-Martin se situe à 0,77 tandis que chez le corégone des autres tributaires du lac Winnipeg et du petit lac Playgreen, elle se situe à 0,46. Deux loci de l'isocitrate déshydrogénase et un locus du lactate désydrogénase sont aussi polymorphes mais aucune différence significative entre la fréquence des allèles n'a été observée à ces loci chez les échantillons de géniteurs susmentionnés. Les grands corégones d'élevage, issus d'un stock des lacs Clearwater et William et déversés à l'état d'alevins ou d'oeufs embryonnés dans la rivière Dauphin et les rapides Grand du lac Winnipeg, ne semblent pas avoir grandement contribué au bagage génétique des stocks locaux de grand corégone si l'on en juge par les différences entre les fréquences des allèles *g-3-pdh-b* et *idh bβ* chez les stocks parentaux et les géniteurs capturés près des sites de lâcher.

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I importance of stock identification to effective fishery management cannot be overemphasized. Stocks of exploited fish species may differ in their capacity to support harvests. Management without regard to stock structure may result in underharvest of some stocks and overharvest of others. Furthermore, failure to recognize their existence can result in the complete elimination of vulnerable stocks and subsequent loss of genetic variability within the population in general (Larkin 1977).

Cushing (1981) describes a stock as a unit of population of lower category than that recognized by taxonomists. According to Booke (1981) a stock is a species group, or population, that maintains or sustains itself over time in a definable area. Ihssen et al. (1981a) describe a stock as an intraspecific group of randomly mating individuals with temporal or spatial integrity. In a broad operational sense a stock is characterized by homogeneity of natural production parameters and may be a portion of a population or include more than one population (Tyler and Gallucci 1980). We prefer to use the term subpopulation in the present study since we endeavour only to provide evidence of restricted gene flow. A subpopulation, as defined by Marr (1957), is a fraction of a population that is itself genetically self-sustaining. It is similar to the local population or deme of Mayr (1963). Future investigations of natural production parameters and the spatial and temporal distribution of lake whitefish in L. Winnipeg may indeed reveal that some or all of our subpopulations are synonymous with stocks.

Lake Winnipeg has supported a commercial fishery for lake whitefish since 1883 and for over 50 yr produced an average of 1 360 000 kg annually (Hewson 1959a). The annual catch has fluctuated from a high of 3.4 million kg in 1904 to a low of 0.3 million kg in 1936 (Hewson 1960). Davidoff et al. (1973) reported that the largest catches of the 10 yr preceeding 1973 barely exceeded the smallest catches in former years, except for 1936. They suggested that the optimum rate of fishing had been exceeded and that the fishery was largely responsible for the depleted condition of whitefish stocks in the lake. The lake was closed to all commercial fishing for one season (1970–71) when high levels of mercury were detected in walleye from some parts of the lake. It was opened experimentally in 1971/72 and annual production climbed steadily from 0.6 million kg that year to a peak of 1.8 million kg in 1980/81 (R. Peters, Manitoba Department of Natural Resources, Box 20, 1495 St. James St., Winnipeg, Man. R3H 0W9, pers. comm.). The

1987/88 harvest was reported to be 1.6 million kg (A. Peterson, Manitoba Department of Natural Resources, Box 1800, Gimli, Man. R0C 1B0, pers. comm.). If this valuable resource is to be maintained and harvested in an optimum manner, stock identification must necessarily be included in future management strategies.

Many methods available for stock identification of fishes have been reviewed by Ihssen et al. (1981a). These authors have emphasized the importance of molecular methods for stock identification although they acknowledge that morphological and meristic data as well as many other characters each have particular advantages in specific circumstances. Past tagging studies (Kennedy 1954; Pollard 1973) suggest that subpopulations of lake whitefish exist in L. Winnipeg. The present study uses a combination of biochemical genetic, morphometric, and meristic characters to investigate the existence of subpopulations of lake whitefish in this lake. The hypothesis to be tested is that all samples of lake whitefish taken from L. Winnipeg during this study are equivalent to samples from a single panmictic stock.

Hatchery-reared lake whitefish fry have been released into L. Winnipeg at Grand Rapids and Dauphin R. over many years. We have attempted to evaluate the success of these plantings through comparisons of allele frequencies among parent stock from the two hatchery lakes and samples of spawners taken at Grand Rapids and Dauphin R. The hypothesis to be tested is that allele frequencies in fish taken at the fry release sites should resemble the allele frequencies of the corresponding parental populations if the introductions have made a detectable genetic contribution to whitefish populations at these sites.

Materials and Methods

Specimen Collections

Lake whitefish were collected from spawning assemblages at seven locations in L. Winnipeg as well as from L. St. Martin and Little Playgreen L., during October and November, 1975 (Fig. 1). Samples were taken from the latter two lakes because tagging studies have shown that lake whitefish move between these lakes and L. Winnipeg. Spawners were also collected from Clearwater (Atikameg) L. and William L. because spawn collected from these lake whitefish populations has been used to supply the hatcheries at Dauphin R. and Grand Rapids. A total of 1 121 fish were collected. Sample sizes ranged from 48 to 205.

Most of the specimens were taken with gillnets although pound nets and beach seines were also used. As soon as possible after capture the fish were placed in individual plastic bags, frozen and then shipped to the Freshwater Institute in Winnipeg and stored at −45°C until examined.

Biochemical Analyses

Phenotypes of the various electrophoretic forms of glycerol-3-phosphate dehydrogenase (G-3-PDH) and lactate dehydrogenase (LDH) were visualized as described by Clayton et al. (1973), and Clayton and Franzin (1970), respectively. Isocitrate dehydrogenase (IDH) phenotypes were also determined according to established electrophoretic methods (Clayton et al. 1971; Clayton and Tretiak 1972). Tissue extracts were prepared for electrophoresis by homogenization of liver tissue with a 0.25 M sucrose solution that also contained 300 mg·L^{-1} nicotinamide adenine dinucleotide phosphate (NADP) in the ratio 1 *g* tissue/2 mL extraction solution. Electrophoresis was conducted at pH 8.0 with a Tris-citrate buffer system (Clayton and Tretiak 1972). After electrophoresis, lake whitefish liver IDH phenotypes were visualized by staining with a solution that contained DL-sodium isocitrate, 150 mg; NADP, 30 mg; Nitroblue tetrazolium, 20 mg; phenazine methosulphate, 30 mg; and $MgCl_2 \cdot 6H_20$, 50 mg; all dissolved in 100 mL of 0.15 M Bicine, pH 8.5 (NaOH).

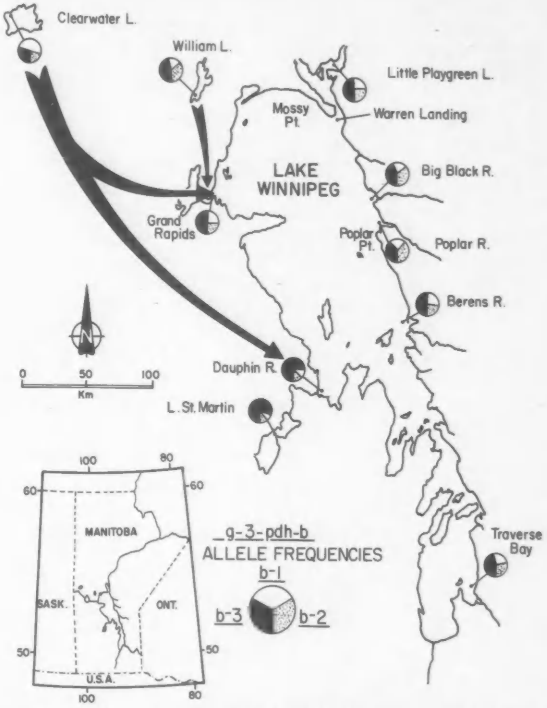

FIG. 1. Locations of samples for lake whitefish spawners during October and November, 1975 with frequencies of *g-3-pdh-b* alleles within L. Winnipeg and four adjacent lakes. Arrows indicate sources of fertilized whitefish eggs and release sites of hatchlings into L. Winnipeg.

For LDH we follow the nomenclature of Bailey et al. (1976). Recent publications (van Beneden et al. 1981; Gonzalez–Villasenor and Powers 1985, 1986) show clearly that the fish *Fundulus heteroclitus* expresses three genes for IDH. In *Fundulus* these authors identified the major liver isozyme as a cytoplasmic IDH-B2. We propose that this system of nomenclature be combined with that of Bailey et al. (1976) whereby the duplicated liver IDH genes in salmonids are identified as *idh Bα* and *idh Bβ*. We further propose, in the interest of clarity, that alleles be numbered in order of increasing anodal electrophoretic mobility. Thus we recommend that the IDH loci previously identified as IDH-3 and IDH-4 (Casselman et al. 1981; Ihssen et al. 1981b) now be designated *idh Bα* and *idh Bβ*, respectively. The alleles IDH-4-100 and IDH-4-119 similarly are now identified as *idh Bβ*-1 and *idh Bβ*-2.

Allele frequencies for *g-3-pdh-b*, *ldh Hα*, *idh Bα* and *idh Bβ* were calculated for samples from each location. Sample sizes ranged from 24 to 59. The calculated Castle-Hardy-Weinberg distribution of phenotypes was compared with observed phenotypic distributions (χ^2 test) to determine homogeneity within samples (Marr and Sprague 1963). Sample allele frequencies were compared for homogeneity among locations using a *G*-test based on maximum likelihood ratios (Sokal and Rohlf 1969).

Phenotypic Analyses

Eight counts and 23 measurements were taken from fish from all locations except Clearwater L. and William. L.; the hatchery stock lakes, where only biochemical analyses were done. A subsample of 48 fish, selected to maximize the range of sizes amongst the available fish, was taken from each location sample. These subsamples also included equal numbers of males and females where possible. Scale samples for age estimation were taken from a position on the left side of each fish just below the origin of the dorsal fin but above the lateral line. Scale ages were determined by Mrs. D. Barnes, an experienced scale reader formerly employed at the Freshwater Instititue in Winnipeg. Following are mean fork lengths (mm), scale ages (yr), and ranges for the subsamples used:

Location	Length	Age
Traverse Bay	461 (397–535)	6.9 (4–9)
Berens R.	372 (315–465)	4.3 (3–8)
Poplar R.	423 (321–515)	6.1 (3–10)
Big Black R.	425 (371–514)	6.1 (3–10)
Dauphin R.	424 (328–496)	6.0 (3–7)
L. St. Martin	400 (307–477)	5.2 (3–9)
Grand Rapids	408 (358–534)	4.6 (3–9)
Little Playgreen L.	432 (366–500)	6.8 (5–9)

The following straight line measurements were taken from the left side of each thawed specimen using a measuring board graduated to 1 mm: fork length, head length, pectoral fin length, pectoral fin origin, pelvic fin length, pelvic fin origin, and pre-postorbital distance after Lindsey (1963). Standard length was measured according to Lindsey (1962). Additional measurements made included adipose fin origin, from the tip of the snout to the origin of the adipose fin; anal fin origin, from the tip of snout to the anterior face of the anal fin with fin extended; dorsal fin origin, from the tip of the snout to the anterior face of the dorsal fin with fin extended. Other measurements, such as the distance from the dorsal fin origin to adipose fin origin and pelvic fin origin to anal fin origin, were derived by calculation. Body length, a measure of the size of the fish, was derived by subtracting head length from fork length.

The following measurements were taken with dial calipers graduated to 0.1 mm: adipose fin length, caudal peduncle depth, gill raker length, interorbital width, maxillary width, and maxillary length (Lindsey 1962); head depth (Lindsey 1963); anal depth and body width (Loch 1974).

Meristic counts were taken with the naked eye, on the left side of the specimen where possible. Where scales were missing, scales pockets were counted. Counts taken included: dorsal fin ray count, caudal peduncle scale count, and suprapelvic scale count (Lindsey 1962); lateral line scales and pectoral fin ray number (Hubbs and Lagler 1964); scales above the lateral line (Kliewer 1970). As well, determinations were made of the number of anal fin rays (only principal rays and excluding anterior rays less than three quarters the length of the longest ray were counted, also the last two rays arising from a common base were counted as one), and gill rakers on the first left gill arch after it was removed in its entirety from the fish, including all bony rudiments. The number of lateral line scales along the standard length was counted starting at the first pored scale touching the pectoral girdle up to and including the last scale anterior to the point marking the end of the standard length.

All morphometric data were transformed ($\log_{10}$) and regressed against body length, using Biomedical Computer Program BMDP6D (Dixon 1975) to determine the effect of size on the body measurement. To remove variations due to differences in fish size within samples the following allometric formulae (Thorpe 1975) were used:

$$\hat{\hat{Y}}_i = 10^{\hat{Y}_i}$$

where

$$\hat{Y}_i = \log Y_i - b\,(\log X_i - \log \bar{\bar{X}})$$

and $\hat{\hat{Y}}_i$ is the adjusted character value of the *i*th specimen; $\hat{Y}_i$ is the logarithm of the adjusted character value of the *i*th specimen; Y_i is the original unadjusted character value of the *i*th specimen; X_i is the body length of the *i*th specimen; $\bar{\bar{X}}$ is the grand mean of the body lengths across all specimens. In our case, 345 mm was the grand mean. The range of sample sizes from all locations overlapped at this value, thus no extrapolation was done. The allometric coefficient *b* is the slope of the relationship between log *Y* and log *X*. We used the individual–within groups relationship (Reist 1986) to calculate allometric coefficients. It is important to note that the nature of this adjustment procedure does not allow future classification of cases of unknown origin since a priori knowledge of sample location is necessary to use the appropriate allometric coefficient.

Correlation analysis (Sokal and Rohlf 1969) was used to test for the linear association between fish size and meristic counts.

Morphometric data were then combined with meristic data and compared between locations using BMDP7M, a stepwise discriminant function analysis program (Dixon 1975). The analysis was not forced to completion. Variables were included in the function using BMDP7M default criteria (*F*-to-enter = 4.0) and a jackknife classification was used to reduce bias. A chance–corrected procedure (Titus et al. 1984) was applied to the results of the discriminant function analysis to determine whether correct classifications were significantly better than by chance alone.

Results

Biochemical Analyses

Observed G-3-PDH-B phenotype distributions were in agreement with the expected Castle–Hardy–Weinberg distribution for samples from all locations except Warren Landing. The χ^2 statistic calculated for this sample was highly significant ($P<0.001$) suggesting that the sample was not comprised of fish from a homogeneous population. Hence these fish were excluded from further analyses.

Results of the G-3-PDH analyses (Table 1) show that while the *g-3-pdh-a-2* allele predominated in all samples, there was considerable variation in allele frequencies at the *g-3-pdh-b* locus (Fig. 1). An a priori test for homogeneity at this locus among all samples from L. Winnipeg, L. St. Martin, and Little Playgreen L. was highly significant (Table 2). Consequently, a posteriori tests were conducted to determine which samples differed. The samples from Dauphin R. and L. St. Martin had similar *g-3-pdh-b-3* allele frequencies (Fig. 1, Table 1) which were also the highest among the samples compared. As shown in Table 2, the test for homogeneity among these samples was

TABLE 1. Frequencies of alleles at glycerol-3-phosphate loci, (*g-3-pdh-a* and *g-3-pdh-b*) in samples of lake whitefish from L. Winnipeg and other lakes.

Location	Allele frequencies					No. fish
	a-1	*a*-2	*b*-1	*b*-2	*b*-3	
Traverse Bay (L. Winnipeg)	0.073	0.927	0.250	0.312	0.438	48
Berens R. (L. Winnipeg)	0.034	0.966	0.254	0.221	0.525	59
Poplar R. (L. Winnipeg)	0.009	0.991	0.222	0.380	0.398	54
Big Black R. (L. Winnipeg)	0.046	0.954	0.241	0.324	0.435	54
Little Playgreen L.	0.019	0.981	0.260	0.260	0.480	52
L. St. Martin	0.060	0.940	0.100	0.110	0.790	50
Dauphin R. (L. Winnipeg)	0.031	0.969	0.135	0.125	0.740	48
Grand Rapids (L. Winnipeg)	0.038	0.962	0.255	0.245	0.500	53
William L.	0.010	0.990	0.170	0.390	0.440	50
Clearwater L.	0.000	1.000	0.500	0.200	0.300	55

TABLE 2. Results of homogeneity tests (*G*-test) of *g-3-pdh-b* allele frequencies among samples of lake whitefish from L. Winnipeg and two adjoining lakes (L. St. Martin and Little Playgreen L.).

Comparison	df	*G*[a]	Significance[b]
a priori			
All samples	14	68.098	***
a posteriori			
(i) Dauphin R. and L. St. Martin	2	0.782	NS
(ii) all samples excluding (i)	10	9.664	NS
(iii) combined (i) and combined (ii)	2	57.652	***
TOTALS	14	68.098	

[a] Distribution of *G* approximated by χ^2-distribution (Sokal and Rohlf 1969).
[b] NS, nonsignificant at the 5% level; ***, significant at the 0.1% level.

nonsignificant. There was also nonsignificance among the remaining samples from L. Winnipeg, and Little Playgreen L. However, the test for homogeneity among the combined groups, Traverse Bay–Berens R.–Poplar R.–Big Black R.–Grand Rapids–Little Playgreen L. and Dauphin R.–L. St. Martin, was highly significant.

Independent comparisons among hatchery lakes and fry release sites revealed significant differences in *g-3-pdh-b* allele frequencies. For Clearwater L. and Dauphin R., $P<0.001$; for Clearwater L. and Grand Rapids, $P<0.001$. A marginal difference ($P \simeq 0.05$) was found between William L. and Grand Rapids.

Observed LDH Hα phenotype distributions agreed with the expected Castle–Hardy–Weinberg distribution for each sample. However, lactate dehydrogenase allele frequencies did not differ significantly among locations. The $ldh\ H\alpha-1$ allele predominated with an overall frequency of 0.95.

The observed IDH phenotype distributions at both loci satisfied Castle–Hardy–Weinberg equilibrium for each sample. The liver isocitrate dehydrogenase genes were polymorphic with one exception, namely the William L. sample which appeared to be fixed for $s\text{-}idh\ B\beta-1$. Statistically, there were no significant differences between all samples at the $idh\ B\alpha$ locus. The overall allele frequencies for the pooled samples were: $idh\ B\alpha-2 = 0.73$, $idh\ B\alpha-3 = 0.27$. However, there was a difference ($P<0.05$) between samples from Grand Rapids and Clearwater L. at the $idh\ B\beta$ locus. Allele frequencies were: Grand Rapids $idh\ B\beta-1 = 0.94$, $idh\ B\beta-2 = 0.06$; Clearwater L. $idh\ B\beta-1 = 0.81$, $idh\ B\beta-2 = 0.19$. The overall allele frequencies for the other samples were: $idh\ B\beta-1 = 0.89$, $idh\ B\beta-2 = 0.11$.

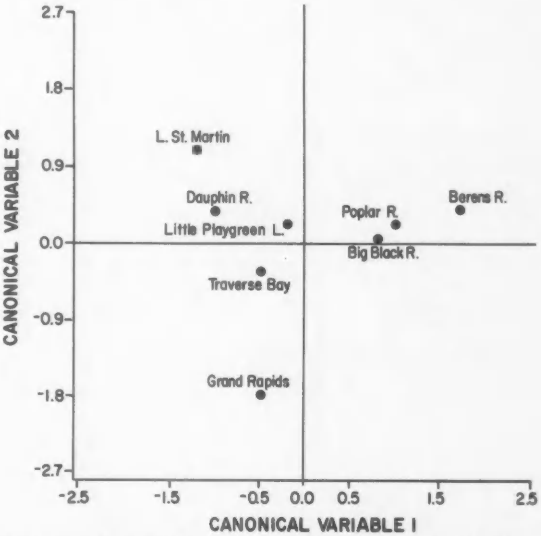

FIG. 2. Plot of canonical variables evaluated at group means from stepwise discriminant function analyses of morphological characters of lake whitefish from L. Winnipeg and two adjoining lakes.

Phenotypic Analyses

All meristic counts were independent of fish size (for H_o: $\rho=0$, $P>0.05$) but all morphometric measurements were positively correlated with fish size. In 76% of cases (122 of 160 regressions) $r \geq 0.8$ and in 89% of cases $r \geq 0.7$.

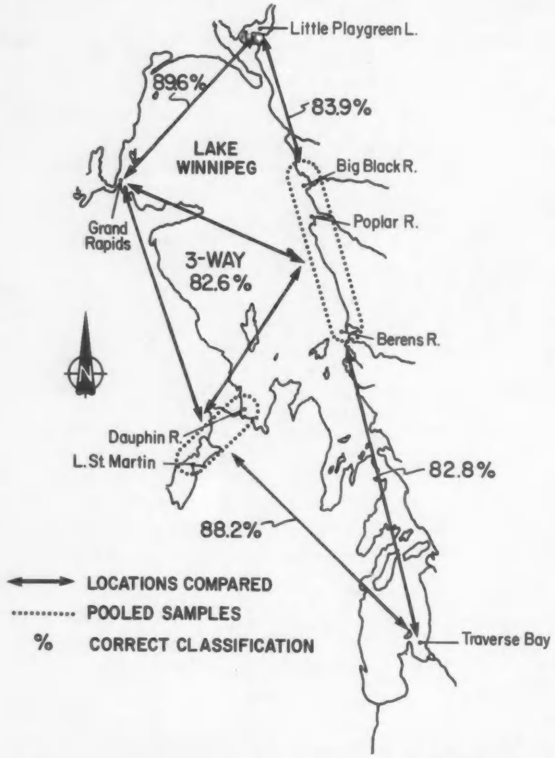

FIG. 3. Effective discrimination between groups of lake whitefish in or near L. Winnipeg based on stepwise discriminant function analyses of morphological characters.

Discriminant function analysis of the morphometric and meristic data (Table 3) shows that overall discrimination by sample location was poor (46%), assuming that each sample location represented a separate subpopulation. Using percent correct classification of samples according to location of capture as a measure of effective discrimination, the best results were from L. St. Martin (60%) and the worst from Big Black R. (27%). Closer examination of Table 3 reveals that, in some cases, most of the mistaken classification was amongst geographically adjacent sites, particularly Berens R.–Poplar R.–Big Black R. and Dauphin R.–L. St. Martin. A plot of canonical variables evaluated at group means (Fig. 2) provides a measure of morphological similarity of the samples. Morphologically similar samples which were geographically adjacent were then pooled, whereby five groups remained. The discriminant analysis was then used to compare these five groups. Subsequent comparisons were made only between adjacent groups (Fig. 3) because detectable differences between such groups provide evidence of segregation. The best discrimination, between Grand Rapids and Little Playgreen L., was nearly 90%. When Little Playgreen L. was compared with Berens R.–Poplar R.–Big Black R., 83.9% of cases were correctly classified. A three-way comparison between Grand Rapids, L. St. Martin–Dauphin R., and Berens R.–Poplar R.–Big Black R. allowed 82.6% correct classification. Traverse Bay versus L. St. Martin–Dauphin R. produced 88.2% correct classification and when Traverse Bay was compared with Berens R.–Poplar R.–Big Black R., 82.8% of cases were correctly classified according to location of capture.

Results of the chance-corrected procedure applied to the discriminant function analysis are shown in Table 4. Kappa (*K*) provides a measure of the improvement in classification over chance. As described by Titus et al. (1984) a kappa of zero indicates that the discriminant analysis has provided no improvement over correct classification by chance alone. A kappa of one indicates perfect agreement. Our data show that, in each case, kappa was lower than the observed agreement (P_o), indicating that some samples were correctly classified by chance alone. However, all cases show correct classification rates that are significantly higher than by chance alone. A kappa only accompanied by a relatively narrow confidence interval, supports the results of the classification table. This appears to be the case with our data with the possible exceptions of Berens–Poplar–Black vs. Playgreen (83.9%) and Traverse vs. Berens–Poplar–Black (82.8%) which could be somewhat inflated.

Overall, two of the eight meristic counts and seven of the 20 morphometric measurements contributed most to the discrimination (Table 5). The Traverse Bay group was characterized by large body proportions such as pectoral fin origin, pectoral fin length, interorbital width, and head length as well as by a high number of lateral line scales. The Berens R.–Poplar R.–Big Black R. group was distinguished by relatively small body measurements such as pectoral fin length and head depth.

TABLE 3. Assignment of lake whitefish into groups using stepwise discriminant function analysis of morphological characters. Note specimens frequently misclassified as belonging to adjacent locations at Berens R.–Poplar R.–Big Black R. and L. St. Martin–Dauphin R.

	Location of capture							
Group assigned to using discriminant analysis	Traverse Bay	Berens R.	Poplar R.	Big Black R.	Little Playgreen L.	L. St. Martin	Dauphin R.	Grand Rapids
Traverse Bay	22	1	1	9	4	5	5	5
Berens R.	2	27	9	9	5	0	1	2
Poplar R.	2	8	20	10	2	1	1	1
Big Black R.	6	6	11	13	2	0	2	2
Little Playgreen L.	5	5	2	3	20	5	7	6
L. St. Martin	3	0	3	1	3	29	12	4
Dauphin R.	3	0	1	1	9	7	17	1
Grand Rapids	5	1	1	2	3	1	3	27
Total No. Fish	48	48	48	48	48	48	48	48

TABLE 4. Results of a chance–corrected procedure applied to the discriminant function analysis of lake whitefish morphological characters.

Groups compared	Correct classification by chance (P_c)	Observed agreement (P_o)	Improvement in classification over chance (κ)	Significance of standard error of κ	95% Confidence interval
All groups (48 × 8)[a]	0.125	0.456	0.378	***[b]	0.320–0.436
Berens–Poplar–Black vs. Dauphin–L. St. Martin vs. Grand Rapids (144, 96, 48)	0.385	0.816	0.701	***	0.664–0.775
Grand Rapids vs. Playgreen (48, 48)	0.500	0.896	0.792	***	0.668–0.916
Berens–Poplar–Black vs. Playgreen (144, 48)	0.597	0.839	0.600	***	0.468–0.732
Traverse vs. Berens–Poplar–Black (48, 144)	0.623	0.828	0.544	***	0.400–0.688
Traverse vs. Dauphin–L. St. Martin (48, 96)	0.553	0.882	0.736	***	0.616–0.856

[a] Group sample sizes.

[b] ***, significant at the 0.1% level.

TABLE 5. Mean values of most useful discriminators used in discriminant function analyses, based on regression of measurements adjusted to a fish of standard size (345 mm in body length). Morphometric measurements are expressed in millimeters and transformed to $\log_{10}$. Standard error in brackets.

	Discriminator								
Location	Pectoral fin length	Lateral line scales	Pectoral fin origin	Prepostorbital length	Body width	Head depth	Caudal peduncle scales	Head length	Interorbital width
Little Playgreen L. ($N=48$)	1.8600 (0.0038)	73.6 (0.42)	1.8415 (0.0044)	1.5410 (0.0042)	1.7381 (0.0030)	1.7708 (0.0032)	23.9 (0.12)	1.8654 (0.0031)	1.3808 (0.0029)
Grand Rapids ($N=48$)	1.8545 (0.0040)	79.4 (0.49)	1.8264 (0.0053)	1.5327 (0.0055)	1.7393 (0. 0038)	1.7870 (0.0041)	25.0 (0.12)	1.8639 (0.0041)	1.3836 (0.0030)
Berens R.–Poplar R.–Big Black R. ($N=144$)	1.8266 (0.0023)	76.1 (0.23)	1.8169 (0.0026)	1.5057 (0.0029)	1.7318 (0.0020)	1.7462 (0.0019)	23.8 (0.08)	1.8401 (0.0020)	1.3666 (0.0017)
L. St. Martin–Dauphin R. ($N=96$)	1.8712 (0.0023)	73.9 (0.34)	1.8369 (0.0025)	1.5236 (0.0030)	1.7119 (0.0025)	1.7651 (0.0023)	24.1 (0.12)	1.8644 (0.0021)	1.3775 (0.0020)
Traverse Bay ($N=48$)	1.8616 (0.0053)	78.1 (0.61)	1.8610 (0.0038)	1.5459 (0.0052)	1.7224 (0.0030)	1.7570 (0.0035)	24.0 (0.14)	1.8690 (0.0038)	1.3736 (0.0037)

L. St. Martin–Dauphin R. fish had long pectoral fins and narrow bodies. The Grand Rapids group had the highest mean numbers of lateral line scales and caudal peduncle scales. Little Playgreen L. fish were distinguished by large body proportions such as pectoral fin length, prepostorbital distance and head depth.

Combinations of effective discriminators varied, depending upon which locations were compared. However, lateral line scales contributed to discrimination in all five comparisons shown in Fig. 3. It was selected first in two, second in two and third in one. As a result, the same comparisons were made using only mean number of lateral line scales (*t*-test). The means (Table 5) all differed ($P<0.001$; $0.001<P<0.005$ for Berens–Poplar–Black vs. Traverse) between the geographically adjacent locations. Within the pooled samples (Table 5), Dauphin R. and L. St. Martin were similar ($P>0.05$) as were Poplar R. and Big Black R. ($P>0.05$). However, the mean number of lateral line scales at Berens R. (74.8) differed ($P<0.001$) from that at Poplar R. (76.5) and Big Black R. (76.9). By itself, Berens R. differed ($P<0.001$) from all geographically adjacent locations except Dauphin R. ($P>0.05$).

Discussion

Biochemical Characterization

The distribution of *g-3-pdh-b* allele frequencies indicates that at least two subpopulations of lake whitefish reside in L. Winnipeg. The subpopulation spawning in L. St. Martin–Dauphin R. is characterized by a *g-3-pdh-b-3* allele frequency of 0.77 while the corresponding frequency is 0.46 for whitefish in the remainder of the lake. Although it is difficult to determine what may have caused this difference in allele frequencies it seems reasonable to conclude that this difference is a reflection of restricted gene flow between the L. St. Martin–Dauphin R. subpopulation and neighbouring populations.

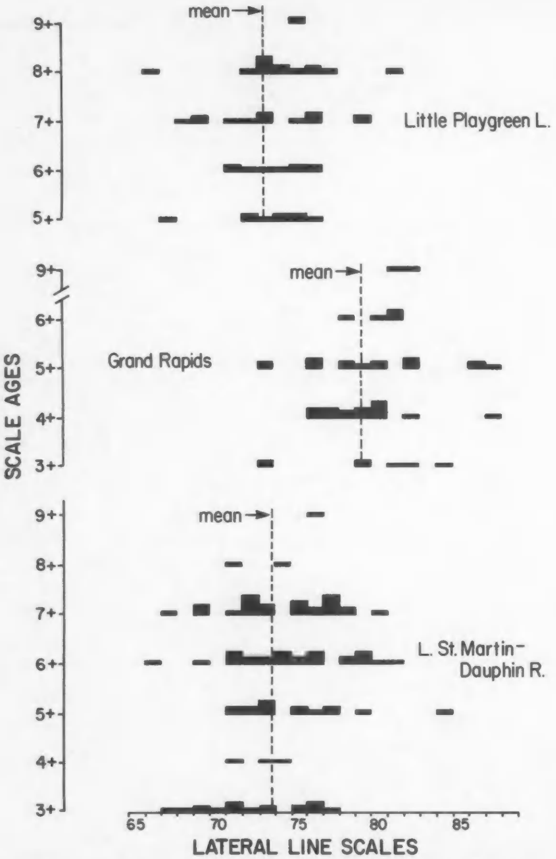

FIG. 4. A comparison of the number of lateral line scales (along the standard length) by age group between samples of lake whitefish from Little Playgreen L., Grand Rapids, and L. St. Martin–Dauphin R.

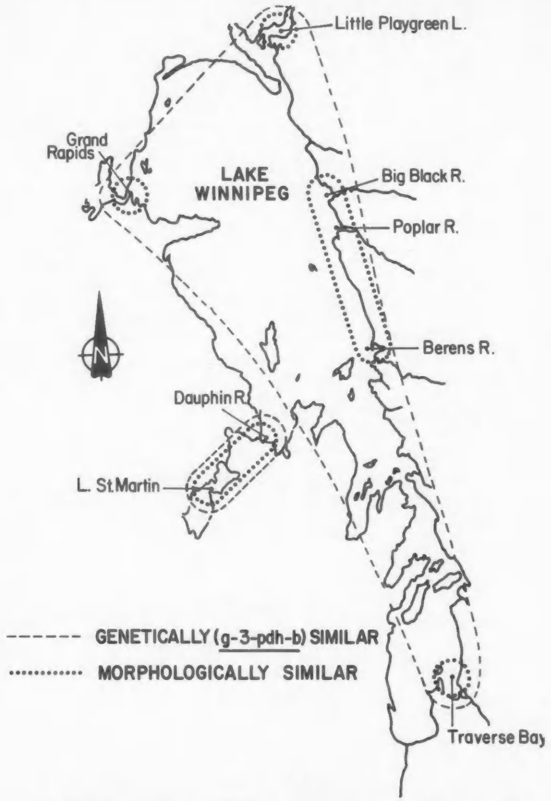

FIG. 5. Subpopulation status of lake whitefish in L. Winnipeg based on biochemical and morphological characters.

One possible cause of the genetic difference between the L. St. Martin–Dauphin R. subpopulation and other L. Winnipeg lake whitefish derives from apparent fluctuations in the reproductive success and size of the subpopulation. Thus the production of lake whitefish in the commercial catch seems to be correlated with water level fluctuation on L. St. Martin such that episodes of low winter water level are followed by poor whitefish production 4 yr later (D. W. Cook, Manitoba Department of Natural Resources, 59 Elizabeth Drive, Thompson, Man. R8N 1X4, pers. comm.). In particular the shallow northeast portion of the lake, which is believed to be a lake whitefish spawning ground, exhibits near zero oxygen levels in the little water that remains under the ice during low water episodes (Anon. 1963). Low water levels during winter in L. St. Martin thus could provide, through the concomitant low levels of dissolved oxygen, a strong selective force which could directly or indirectly affect the *g-3-pdh-b* allele frequency of the lake whitefish. Alternatively, the apparent fluctuations in the size of this lake whitefish subpopulation, as indicated by the variations in the commercial production, may represent a means whereby the frequencies of the *g-3-pdh-b* alleles could drift stochastically.

The χ^2 value ($P<0.001$) which resulted when the samples from Warren Landing was tested for Castle–Hardy–Weinberg equilibrium at the *g-3-pdh-b* locus suggests the existence of at least one other subpopulation of lake whitefish which this study was not able to identify. Equilibrium within the Warren Landing sample was not satisfied due to a heterozygote deficiency. Such a deficiency is consistent with a mixture of fish from subpopulations which differ in allelic frequencies (Wahlund 1928). Movement of lake whitefish is known to take place between L. Winnipeg and Little Playgreen L. (Pollard 1973). In late September and early October 1979, nearly 3 000 lake whitefish were tagged near spawning grounds at the northern end of L. Winnipeg at Mossy Point (Fig. 1). Shortly thereafter that same fall, 16 tagged lake whitefish were recaptured on spawning grounds in Little Playgreen L. A similar pattern was observed when the study was repeated in 1971. Our Warren Landing sample was collected over a period of 4 d from a single gillnet set off Mossy Point, and it could have been comprised of whitefish migrating downstream to spawn in Little Playgreen L. as well as whitefish spawning at Mossy Point. Because there were no significant differences in *g-3-pdh-b* allele frequencies amongst spawners from Grand Rapids, Big Black R. or Little Playgreen L. (Fig. 1, Table 2) the source of the heterogeneity could well be from a genetically distinct (*g-3-pdh-b*) subpopulation spawning at Mossy Point.

Tag recoveries led Pollard (1973) to suspect the existence of a subpopulation spawning in the Mossy Point area. By the end of 1972, 525 whitefish, tagged during the previously mentioned study, had been recovered by fishermen. Of these, 500 were taken in L. Winnipeg and 25 were taken in Playgreen L. The vast majority were recovered within 105 km of the tagging site at Mossy Point. Electrophoretic investigation of spawners from this location should be continued to confirm the existence of this subpopulation.

Although the Warren Landing sample was excluded from further analyses due to the heterogeneity problem, *idh Bα* and *idh Bβ* allele frequencies were determined, to test the genetic model. Observed IDH phenotype distributions at both loci satisfied Castle–Hardy–Weinberg equilibrium. Taken by itself, this could appear to be in contradiction to the nonagreement found at the *g-3-pdh-b* locus previously discussed. However, there were no significant differences in allele frequencies at either of these two *idh* loci between any of the samples from L. Winnipeg, Little Playgreen L., L. St. Martin, or William L. If the suspected subpopulation spawning at Mossy Point also shared the common *idh Bβ* allele frequencies, equilibrium would be expected regardless of whether the sample was a mixture of two subpopulations or not.

Morphological Characterization

Morphological differences among L. Winnipeg lake whitefish are sufficient to suggest their separation into at least five subpopulations. These are: Traverse Bay, L. St. Martin–Dauphin R., Berens R.–Poplar R.–Big Black R., Grand Rapids, and Little Playgreen L. Although correct classification of lake whitefish by location of capture was poor (46%) when all groups were compared (Table 4), there was a significant improvement in classification over chance (38%). We interpret this to mean that group structure exists within L. Winnipeg, based on morphological differences. The pooling of morphologically similar groups resulted in a marked improvement in correct classification, even when the results were corrected for chance. A kappa much lower than P_o suggests that correct classification rates are inflated (Titus et al. 1984). For our data, kappa was not much lower than P_o (Table 4) except for the comparisons between the pooled Berens–Poplar–Black sample and the Playgreen and Traverse samples. In these cases the low κ could be due to unequal sample sizes. According to Titus et al. (1984), as the relative size of a single group become predominant, correct classification by chance alone (P_o) tends to increase towards unity with a corresponding decrease in κ. In practice then, it becomes increasingly difficult to demonstrate significant discrimination power with such data. Nevertheless, our data do show correct classification of a large percentage of cases (i.e. greater than that expected by chance) which we interpret as evidence of group differences.

Bodaly (1977) found that environmental factors modified growth rate and thereby influenced body morphology in sympatric, divergent forms of lake whitefish in some Yukon lakes. Various investigators (Martin 1949; Kliewer 1970) have found faster growing lake whitefish to have smaller heads and fins, shorter snout length, and smaller eyes than their slower growing counterparts. Using relative growth analysis, Martin (1949) concluded that growth rate affects body size at which inflections take place in the rate of growth of a body part relative to the body as a whole. Significant environmental factors which can affect growth rate exist in L. Winnipeg and vary between different parts of the lake. They include temperature (Brunskill et al. 1979), food availability and water chemistry (Rybicki 1966), and rate of exploitation (Hewson 1959a, 1959b, 1960). In Traverse Bay, lake whitefish occur only as an incidental catch in fisheries carried out for other species in the south basin of the lake. After 1954, fishing effort nearshore from Poplar Point to Warren Landing was 12 times as great as in nearshore areas within the remainder of the whitefish grounds.

Many morphological and meristic characters are in fact phenotypic characteristics that may be environmentally modifiable and therefore must be used carefully in subpopulation studies. Horrall (1981) states that stock-isolating mechanisms such as homing and site imprinting in Great Lakes fishes produce stock specific adaptations to the local environment which can involve changes in the morphological, physiological or behavioral characteristics of the fish. If the lake whitefish do indeed home to their natal spawning grounds and inhabit a particular environment, the effects of which manifest themselves phenotypically in successive generations, then assigning subpopulation status on the basis of morphological differences would be justified. For such an analysis to be useful the samples must necessarily be comprised of a number of successive year-classes to provide evidence that morphological differences are indeed consistent within a group over a period of years. Such consistency implies genetic and/or environmental similarity from generation to generation.

The biochemical data provide evidence for a degree of homing in whitefish that is apparently reflected in restricted gene flow between the subpopulation spawning in L. St. Martin–Dauphin R. and other subpopulations. Samples taken during this study were comprised of a minimum of five and a maximum of eight year-classes. Figure 4 shows that there were differences in the number of lateral line scales between groups, yet the number of lateral line scale counts was similar amongst successive ages within each group. Lateral line scales are known to be environmentally modifiable (Svardson 1952). Whether the differences shown in Fig. 4 are due to environmental or genetic factors is not known. Genetic differences (*g-3-pdh-b*) were demonstrated between Grand Rapids and L. St. Martin–Dauphin R.; however, they were not evident between Little Playgreen L. and Grand Rapids based on the enzymes examined in this study.

Whether the causal agent is genetic or environmental, samples from spawning assemblages which show morphological differences between groups but similarity between successive generations within each group do indeed provide evidence of reproductive isolation and hence are useful for stock identification purposes.

Discriminant function analysis is attractive for studies such as ours because it provides a means whereby multiple groups can be compared using multiple variables. Difficulties arise, however, when variables used are correlated with size. Variations in size distribution of individuals among samples can exist, as they did in our study, and if size is not adjusted for, spurious conclusions based on size differences rather than shape differences can be drawn. Reist (1985) studied a variety of univariate transformations that attempt to separate size and shape variation. They include logarithms, ratios, logarithms of ratios, allometric adjustment and regression techniques. He found that those that were at least partly effective in removing size variation affected the correlation and covariance structure of the data in different ways and resulted in different biological interpretations for principal component and discriminant analyses.

In a study of lake whitefish stocks of Lake Huron, Casselman et al. (1981) used ratios of body measurements on fork lengths to remove variations of fish size within each sample. They found discrepancies between the statistical and biological interpretations when dealing with differences among stocks, which led them to suspect the adequacy of their correction technique for discriminant analysis. Reist (1985) found that adjustment of size using a regression technique or allometric adjustment to a standard size was preferred as these techniques allowed for the complete removal of size variation with minimal adverse effects. We used an allometric adjustment to a standard size in our study. Reist (1986) discusses three types of relationships which may be used to calculate allometric coefficients when using an allometric size adjustment. They include a pooled group, which assumes homogeneity of slopes and intercepts, a common-within groups, which adjusts all within-groups lines to a common slope but intercepts may differ, and a separate within-groups relationship where slopes and intercepts may differ for each group. The last two methods require some a priori information as to the grouping structure. We used the separate within-groups relationship in our study with location of capture as the grouping structure. Reist (1986) found that the separate within-groups classification accuracy was better than that for the pooled and common-within data but preferred the use of the common-within groups allometric coefficient for shape estimation because it was better at dissociation of size and shape than the within-group coefficients.

With respect to group differences, we drew virtually the same conclusions from the discriminant function analysis, which included variables correlated with size, and the comparison of mean number of lateral line scales, which was independent of size. Hence our method of adjusting for the size covariate did not result in spurious group differences. In fact, conclusions drawn, based on the discriminant function analysis, may have been somewhat conservative as a result of pooling. We pooled the samples from Berens R., Poplar R., and Big Black R. due to apparent morphological similarities, using all variables. Yet the Berens R. sample differed in mean number of lateral line scales from adjacent Poplar R. and Big Black R. when only this variable was examined. The pooling appears to have masked this difference. Although Berens R. also differed in number of lateral line scales from adjacent Traverse Bay to the south, and Grand Rapids to the north, we are reluctant to assign it subpopulation status based on differences in this one character alone. Its similarity in lateral line scales to Dauphin R. appears to be coincidental because the two differ genetically (Fig. 1). Future investigations should consider the possibility that lake whitefish spawning near Berens R. may indeed represent a separate subpopulation.

Subpopulation Status

Together, the biochemical and morphological evidence provided by this study suggests the existence of at least five subpopulations of lake whitefish in L. Winnipeg (Fig. 5). Biochemistry and morphology have each independently identified the lake whitefish of L. St. Martin–Dauphin R. as a subpopulation. Although the *g-3-pdh-b* allele frequencies calculated for the remainder of L. Winnipeg are similar, the morphological evidence suggests at least four separate subpopulations. It must be remembered that while differences in a character between groups may be taken as evidence of genetic isolation, similarities have no simple interpretation, and certainly do not prove identity. A further example is provided by the marked difference in *g-3-pdh-b* allele frequencies observed between L. St. Martin–Dauphin R. and the remainder of L. Winnipeg which may be contrasted with the insignificant differences found in frequencies of *g-3-pdh-a, ldh hα, idh Bα* and *idh Bβ* between the two groups.

Effect of Hatcheries

Prior to the present sample collection, lake whitefish spawn had been taken for at least 25 yr at Clearwater L., incubated at the Dauphin R. hatchery, and introduced as eyed eggs or fry into L. Winnipeg near the mouth of the Dauphin R. Since 1967, lake whitefish spawn from both Clearwater L. and William L. has been incubated at the Grand Rapids hatchery and introduced as fry into L. Winnipeg at this location (J. Ziemanski, Manitoba Department of Natural Resources, Whiteshell P.O. Man., R0E 2H0, pers. comm.). Our hypothesis is that allele frequencies should be similar between parent stocks and spawners caught at fry release sites if the hatchery-reared fish were making a measurable genetic contribution to local stocks. Allele frequencies at the *g-3-pdh-b* locus differ between lake whitefish samples from Clearwater L. and L. St. Martin–Dauphin R., and in a manner contradictory to what would be expected if hatchery fish from Clearwater L. had contributed genetically to the L. St. Martin–Dauphin R. subpopulation. The former sample has a high frequency (0.50) of the *g-3-pdh-b-1* allele while the latter sample has a high frequency (0.77) of the *g-3-pdh-b-3* allele (Table 1; Fig. 1). Similarly, the high frequency of the Clearwater L. *g-3-pdh-b-1* allele is in contradiction to the high frequency (0.50) of the *g-3-pdh-b-3* allele at Grand Rapids. Additionally, a parallel difference between Clearwater L. and Grand Rapids fish exists at the *idh Bβ* locus. The difference between William L. and Grand Rapids appears to be only marginal and is based on differences in the relative frequencies of the *g-3-pdh-b-1* and *g-3-pdh-b-2* alleles. The nature of these observed differences suggests that we should conclude that introductions of hatchery-reared lake whitefish into L. Winnipeg, from Clearwater L. at least, have not been successful. However, our comparisons are based on relative frequencies of alleles common to both donor and recipient lakes. Thus, there is room for speculation.

Some hatchery-reared fish may indeed have been present in samples from the fry release sites but they would be impossible to identify individually. Additionally, we have no knowledge of allele frequencies of spawners at Dauphin R. and Grand Rapids prior to any hatchery introductions. Did the difference noted in this study between Dauphin R. and the other L. Winnipeg samples exist before hatchery operations began? If so, then it was obviously not caused by hatchery-reared fish. If not, it could have been influenced by hatchery fish from Clearwater L. although it seems unlikely given the marked differences in allele frequencies we noted between the two locations.

The *g-3-pdh-b* allele frequencies at Grand Rapids are similar to most of the other L. Winnipeg samples. If this was the case before hatchery plantings began at this site, which seems most likely, introductions of hatchery-reared fish from Clearwater L. have not been sufficient to change the Grand Rapids frequencies from the remainder of L. Winnipeg in spite of the differences in allele frequencies between Clearwater L. and L. Winnipeg. If the allele frequencies were different between Grand Rapids and L. Winnipeg before the hatchery plantings began, it seems unlikely that Clearwater L. introductions influenced them to the extent that they are now similar to L. Winnipeg. The latter case could hold for William L., however, because its *g-3-pdh-b* allele frequencies are more similar to Grand Rapids than are those of Clearwater L. The problem is that the similarity in *g-3-pdh-b* allele frequencies between William L. and L. Winnipeg in general, and the lack of knowledge of prehatchery allele frequencies at Grand Rapids, make it impossible to demonstrate hatchery success in this case using this technique.

It appears, then, that if relative frequencies of alleles are to be used as a measure of the success of hatchery plantings, results will be more conclusive if allele frequencies of spawners in the vicinity of proposed release sites are known prior to any hatchery introductions and that these allele frequencies differ significantly with those of the parent stock at the outset. Of course, much better evidence for the success of hatcheries would come from the introduction of hatchery-reared fish with an endemic allele.

Implications for Fishery Management

Effective stock identification must start with: (1) recognition of the presence of subpopulations; (2) location of the spawning grounds of the different subpopulations and; (3) delineation of the geographic range and the spawning range of each subpopulation (MacLean and Evans 1981; Marr and Sprague 1963). The present study has, in part, achieved the first two objectives. Delineation of the geographic range and spawning range is next. A start has been made. Cook and MacKenzie (1979) provide evidence from tagging studies (1974–76) that lake whitefish spawners tagged at the mouth of the Dauphin R. were recaptured on the spawning grounds in L. St. Martin. Similarly, lake whitefish tagged on the spawning grounds in L. St. Martin were subsequently recaptured in L. Winnipeg. This suggests that the spawning range of this subpopulation could include the Dauphin R. area and L. St. Martin. Most recaptures by the commercial fishery were within 70 km of the tagging site suggesting that the geographic range of this subpopulation may be somewhat localized.

Probably the distributions of some lake whitefish stocks in L. Winnipeg overlap during part of the year. Collection of vital statistics from such admixtures is meaningless from a population dynamics point of view. Also, the extent of fishing on the stocks cannot be determined unless the relative contributions of each stock to the total harvest is known. Tagging on the spawning grounds and subsequent mixture of tags recovered during the commercial fishery provides evidence of such intermixing as well as a measure of the relative contributions of stocks to the harvest. If mixing is the case, vital statistics should be collected by sampling lake whitefish on the spawning grounds. If the significant differences in exploitation referred to by Hewson (1960) over parts of the lake still exist, different stocks could be subject to varying fishing pressure. Failure to recognize this will result in less than optimum utilization of the resource.

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References

ANONYMOUS. 1963. Pollution investigations 1962–63. Fishing, a Bulletin for Commerical Fishermen 3: 24–25.

BAILEY, G. S., H. TSUYUKI, AND A. C. WILSON. 1976. The number of genes for lactate dehydrogenase in salmonid fishes. J. Fish. Res. Board Can. 33: 760–767.

BODALY, R. A. 1977. Evolutionary divergence between currently sympatric lake whitefish *Coregonus clupeaformis*, in the Yukon Territory. Ph.D. thesis. University of Manitoba, Winnipeg, Man. 119 p.

BOOKE, H. E. 1981. The conundrum of the stock concept — are nature and nurture definable in fishery science? Can. J. Fish. Aquat. Sci. 38: 1479–1480.

BRUNSKILL, G. J., P. CAMPBELL, AND S. E. M. ELLIOT. 1979. Temperature, oxygen, conductance and dissolved major elements in Lake Winnipeg. Can. Fish. Mar. Serv. Manuscr. Rep. 1526: v + 127 p.

CASSELMAN, J. M., J. J. COLLINS, E. J. CROSSMAN, P. E. IHSSEN, AND G. R. SPANGLER. 1981. Lake whitefish (*Coregonus clupeaformis*) stocks of the Ontario waters of Lake Huron. Can. J. Fish. Aquat. Sci. 38: 1772–1789.

CLAYTON, J. W., AND W. G. FRANZIN. 1970. Genetics of multiple lactate dehydrogenase isozymes in muscle tissue of lake whitefish (*Coregonus clupeaformis*). J. Fish. Res. Board. Can. 26: 1115–1121.

CLAYTON, J. W., W. G. FRANZIN, AND D. N. TRETIAK. 1973. Genetics of glycerol-3-phosphate dehydrogenase isozymes in white muscle of lake whitefish (*Coregonus clupeaformis*). J. Fish. Res. Board Can. 30: 187–193.

CLAYTON, J. W., AND D. N. TRETIAK. 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. J. Fish. Res. Board Can. 29: 1169–1172.

CLAYTON, J. W., D. N. TRETIAK, AND A. H. KOOYMAN. 1971. Genetics of multiple malate dehydrogenase isozymes in skeletal muscle of walleye (*Stizostedion vitreum vitreum*). J. Fish. Res. Board Can. 28: 1005–1008.

COOK, D., AND J. MACKENZIE. 1979. Lake St. Martin whitefish study. Manit. Dep. Mines Nat. Resour. Environ. MS Rep. 79–12: 40 p.

CUSHING, D. H. 1981. Fisheries biology, a study in population dynamics. University of Wisconsin Press, Madison, WI. 295 p.

DAVIDOFF, E. B., R. W. RYBICKI, AND K. H. DOAN. 1973. Changes in the population of lake whitefish (*Coregonus clupeaformis*) in Lake Winnipeg from 1944 to 1969. J. Fish. Res. Board Can. 30: 1667–1682.

DIXON, W. J. 1975. BMDP, Biomedical Computer Programs. University of California Press, Berkeley, CA. 792 p. (revised August 1976).

GONZALEZ–VILLASENOR, L. I., AND D. A. POWERS. 1985. A multilocus system for studying tissue and subcellular specialization: the three NADP-dependent isocitrate dehydrogenase isozymes of the fish *Fundulus heteroclitus*. J. Biol. Chem. 260: 9106–9113.

1986. A multilocus system for studying tissue and subcellular specialization: the pH and temperature dependence of two major NADP-dependent isocitrate dehydrogenase isozymes of the fish *Fundulus heteroclitus*. J. Biol. Chem. 261: 11471–11477.

HEWSON, L. C. 1959a. A seven-year study of the fishery for lake whitefish, *Coregonus clupeaformis*, on Lake Winnipeg. J. Fish. Res. Board Can. 16(1): 107–120.

1959b. A study of six winter seasons of commercial fishing on Lake Winnipeg, 1950–1955. J. Fish. Res. Board Can. 16(1): 131–145.

1960. A history of the Lake Winnipeg fishery for whitefish, *Coregonus clupeaformis*, with some reference to its economics. J. Fish. Res. Board Can. 17(5): 625–639.

HORRALL, R. M. 1981. Behavioral stock-isolating mechanisms in Great Lakes fishes with special reference to homing and site imprinting. Can. J. Fish. Aquat. Sci. 38: 1481–1496.

Hubbs, C. L., and K. F. Lagler. 1964. Fishes of the Great Lakes region. University of Michigan Press, Ann Arbor, MI. 213 p.

Ihssen, P. E., H. E. Booke, J. M. Casselman, J. M. McGlade, N. R. Payne, and F. M. Utter. 1981a. Stock identification: materials and methods. Can. J. Fish. Aquat. Sci. 38: 1838–1855.

Ihssen, P. E., D. O. Evans, W. J. Christie, J. A. Reckahn, and R. L. DesJardine. 1981b. Life history, morphology, and electrophoretic characteristics of five allopatric stocks on lake whitefish (*Coregonus clupeaformis*) in the Great Lakes region. Can. J. Fish. Aquat. Sci. 38: 1790–1807.

Kennedy, W. A. 1954. Tagging returns, age studies and fluctuations in abundance of Lake Winnipeg whitefish, 1931–1951. J. Fish. Res. Board. Can. 11(3): 284–309.

Kliewer, E. V. 1970. Gill raker variation and diet in lake whitefish *Coregonus clupeaformis* in northern Manitoba, p. 147–165. *In* C. C. Lindsey and C. S. Woods [ed.] Biology of Coregonid Fishes. University of Manitoba Press, Winnipeg, Man. 560 p.

Larkin, P. A. 1977. An epitaph for the concept of maximum sustained yield. Trans. Am. Fish. Soc. 106(1): 1–11.

Lindsey, C. C. 1962. Distinctions between the broad whitefish, *Coregonus nasus*, and other North American whitefishes. J. Fish. Res. Board Can. 19: 687–714.

1963. Sympatric occurrence of two species of humpback whitefish in Squanga Lake, Yukon Territory. J. Fish. Res. Board Can. 20: 749–767.

Loch, J. S. 1974. Phenotypic variation in the lake whitefish, *Coregonus clupeaformis*, induced by introduction into a new environment. J. Fish. Res. Board Can. 31: 55–62.

MacLean, J. A., and D. O. Evans. 1981. The stock concept, discreteness of fish stocks, and fisheries management. Can. J. Fish. Aquat. Sci. 38: 1889–1898.

Marr, J. C. 1957. The problem of defining and recognizing subpopulations of fishes, p. 1–6. *In* Contributions to the study of subpopulations of fishes. U.S. Fish Wildl. Serv. Spec. Sci. Rep. 208: 129 p.

Marr, J. C., and L. M. Sprague. 1963. The use of blood group characteristics in studying subpopulations of fishes. Int. Comm. Northwest Atl. Fish. Spec. Publ. 4: 308–313.

Martin, W. R. 1949. The mechanics of environmental control of body form in fishes. Univ. Toronto Stud. Biol. Ser. 58: 1–91.

Mayr, E. 1963. Animal species and evolution. Harvard University Press, Cambridge, MA. 797 p.

Pollard, W. R. 1973. A preliminary evaluation of whitefish movements in the north basin of Lake Winnipeg and between Lake Winnipeg and Playgreen Lake. Manit. Dep. Mines Resour. Environ. Manage. 34 p.

Reist, J. D. 1985. An empirical evaluation of several univariate methods that adjust for size variation in morphometric data. Can. J. Zool. 63: 1429–1439.

1986. An empirical evaluation of coefficients used in residual and allometric adjustment of size covariation. Can. J. Zool. 64: 1363–1368.

Rybicki, R. W. 1966. Limnological survey of the north basin of Lake Winnipeg, 1963 and 1964. Manit. Fish. Branch MS Rep. 71 p.

Sokal, R. R., and F. J. Rohlf. 1969. Biometry. W. H. Freeman and Co., San Francisco, CA. 776 p.

Svardson, G. 1952. The coregonid problem. IV. The significance of scales and gillrakers. Inst. Freshwater Res. Drottningholm. Rep. 33: 204–232.

Thorpe, R. S. 1975. Quantitative handling of characters useful in snake systematics with particular reference to intraspecific variation in the Ringed Snake *Natrix natrix* (L.). Biol. J. Linn. Soc. 7: 27–43.

Titus, K., J. A. Mosher, and B. K. Williams. 1984. Chance-corrected classification for use in discriminant analyses: ecological applications. Am. Midl. Nat. 3: 1–7.

Tyler, A. V., and V. F. Gallucci. 1980. Dynamics of fished stocks. p. 111–147. *In* R. T. Lackey and L. A. Neilsen [ed.] Fisheries Management. John Wiley and Sons, Inc., New York, NY. 422 p.

van Beneden, R. J., R. E. Cashon, and D. A. Powers. 1981. Biochemical genetics of *Fundulus heteroclitus* (L.). III. Inheritance of isocitrate dehydrogenase (*Idh-A* and *Idh-B*) 6-phosphogluconate dehydrogenase (*6-Pgdh-A*), and serum esterase (*Est-S*) polymorphisms. Biochem. Genet. 19: 701–714.

Wahlund, S. 1928. Zusammensetzung von Populationen und Korrelationserscheinungen vom Standpunkt der Vererbungslehre aus betrachtet. Hereditas 11: 65–106.

Ultrastructural Observations on the Ovary and Eggs, and the Development of Egg Adhesion in Pacific Herring (*Clupea harengus pallasi*)

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Changes in Pacific herring ovarian tissues and eggs during the four stages, late vitellogenic, ovulating, ovulated, and fertilized, were investigated by transmission and scanning electron microscopy. During the late vitellogenic and transitional stages the egg membranes consisted of five layers: the externus, distal inter-layer, internus, proximal inter-layer, and the sub-internus. The proximal and distal inter-layers do not appear to have been described elsewhere. A class of small, non-vitellogenic oocytes which were observed in late vitellogenic and ovulating ovaries, but not in ovulated ones or spawned eggs, are tentatively described as 'pre-ovulatory atretic oocytes'. Transmission electron microscopy (TEM) results demonstrated a collapse of the pores in the internus which may serve as a mechanical means of achieving egg membrane semipermeability during the first, or ovulation hydration. A thin, electron-dense layer was deposited on the external surface of the externus during ovulation. This epi-layer expanded on contact with seawater and became strongly adhesive. The results of the analyses are discussed in relation to an industrial roe-product quality problem called 'fragile roe' — an expression which describes a condition of reduced adhesion of eggs.

Les changements qui s'effectuent chez les tissus ovariens et les oeufs du hareng du Pacifique au cours des quatre stades de développement soit la fin de la vitellogénèse, l'ovulation, la post-ovulation et la fertilisation, ont été étudiés au microscope électronique à transmission et à balayage. À la fin de la vitellogénèse et au cours des stades de transition, les membranes de l'oeuf se composent de cinq couches : l'externus, la couche médiane distale, l'internus, la couche médiane proximale et le sub-internus. Les couches médianes distale et proximale ne semblent pas avoir été décrites auparavant. Un type de petits ovocytes libres de vitellus observés dans les ovaires à la fin de la vitellogénèse et au moment de l'ovulation, mais non dans les ovaires après l'ovulation ou chez les oeufs pondus, sont identifiés provisoirement comme des "ovocytes pré-ovulaires atrésiques". Les résultats d'études au microscope électronique à transmission révèlent un affaissement des pores de l'internus qui peut servir comme moyen mécanique d'obtenir la semi-perméabilité de la membrane de l'oeuf au cours de la première hydratation. Une mince couche opaque aux électrons est déposée sur la surface externe de l'externus pendant l'ovulation. Cette épicouche se dilate au contact de l'eau de mer pour devenir nettement adhésive. Les résultats des analyses sont présentés en rapport avec le problème de la "rogue fragile" auquel fait face l'industrie et qui concerne la faible adhésion des oeufs.

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British Columbia's Pacific herring stocks are harvested commercially for two principal roe products. Sac-roe is obtained from sexually mature females harvested by seine and gillnet. Roe-on-kelp is produced by impoundment-confined spawning on suspended *Macrocystis* lamellae, or by suspending the lamellae in areas of active wild spawning. Both products rely on the adhesion of ovulated herring eggs for their character and consumer appeal, and hence marketability. The roe-on-kelp product also requires egg fertilization, with its accompanying second hydration to swell the eggs, "harden" the membrane, and render the product "crunchy" when eaten. Some roe producers have recently complained of "fragile" roes, where adhesiveness is either incompletely developed, or has deteriorated in some way. Further, Pacific herring eggs are normally spawned in inter- and sub-tidal high energy environments. Successful incubation and embryonic development require that eggs adhere to marine macrophytes or to other substrates within these environments. Therefore, to understand roe adhesiveness and fragility, a detailed description of the development and activation of the adhesive mechanism and egg "hardening" during late vitellogenesis, ovulation, and fertilization is required.

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Some relevant work has been conducted on herring by Bowers and Holliday (1961) who produced an overview of both male and female gonad development throughout the herring reproductive cycle. Tsuyuki and Cheng (1985a, 1985b) examined the quality and yield of roe from herring impounded during the latter stages of gonad maturation, and the effects of processing conditions on the development of fragility. Rosenthall and Odense (1986) described micropyles and sperm penetration during fertilization, and Hay et al. (1987) examined ovarian development and oocyte diameter as maturation criteria, and described resting and vitellogenic oocytes. None of this work adequately describes ultrastructural changes that occur in the egg membrane during late development. Accordingly, this scanning and transmission electron microscopic study examines the physical changes that occur in herring ovaries and eggs during the four stages; late vitellogenic, ovulating, ovulated, and fertilized.

Materials and Methods

Fishing, impoundment, and sampling for this project are described in Gillis et al. (1990). Two subsamples of about 1 000 fish each were removed from the master impoundment and housed in two small impoundments of about 30 m^3. The fish were allowed to adjust to their surroundings for 7 d prior to sampling. The fish were not fed throughout the impoundment and sampling period.

According to data from previous years of test fishing, population ovulation was estimated to occur during the first 2 wk of March. Sampling from the small impoundments was conducted on February 16 and 23; March 1, 5, 8, 9, 10, 12, 13, 14, 15, 16, 19, 22, and 29; and April 5 and 12. For each sample, the impoundment web was raised to concentrate the fish near the surface. Forty to 60 fish were dip-netted randomly from the impoundment and transported in plastic pails containing seawater to the laboratory. For late vitellogenic and ovulating fish, gender was determined by examining the gonads directly through a small incision in the lateral body wall. Gender of sexually mature fish was determined by the nature of the gametes that extruded spontaneously from the urogenital papilla.

The four stages examined in this study were recognized as follows: Late vitellogenic ovaries were identified by their size, filling two-thirds or more of the body cavity, their opaque white appearance, obvious vascularization, and the intimate binding of the ovarian stroma with the eggs (intermediate between Hjort (1910) stages IV and V). Ovulating ovaries filled the body cavity. Their eggs developed translucency and dissociated from the degenerating ovarian stroma, (Hjort 1910, stage V). Ovulated ovaries were transparent and large, producing pronounced lateral bulging of the body wall when viewed from the dorsal side and the eggs were large, loose, and running (Hjort 1910, stage VI). Eggs for the fertilized sample were taken from ovulated ovaries and fertilized in a beaker of sea water containing abundant suspended sperm.

To optimize ultrastructural preservation, ovarian tissue and eggs were fixed for both transmission and scanning microscopy by perfusion according to Hinton (1975), modified as follows to suit Pacific herring. The perfusate solution was prepared by dissolving 4.28 g sodium cacodylate and 25 mL of 8% glutaraldehyde in distilled water, bringing the volume to 95 mL. The pH was adjusted to 7.4 with 0.1 N HCl. Distilled water was added to bring the volume to 100 mL. The osmolarity was adjusted to 440 mOs with NaCl (about 0.25 g) to match that measured for herring plasma. For each of the four developmental stages examined, three female herring, anesthetized with CO_2, were killed by striking the head, taking care to avoid rupturing the vascular system. The fish were placed in supine positions in V-shaped grooves carved in styrofoam blocks. The perfusion apparatus consisted of a 50 mL reservoir suspended 1 m above the fish, and connected to 1.5 m of polyethylene tubing (1.22 mm O.D. and 0.76 mm I.D.) which was heat flared at the free end. The heart and ventral aorta were exposed by an incision in the midventral body wall. The flared free end of the cannula was inserted through an incision in the ventricle, into the bulbus. The cannula was secured in the bulbus with a cotton thread. The flow of perfusate was started immediately. Yellowish colouration, characteristic of glutaraldehyde fixation (Hinton, 1975), appearing on the white ventral body surface indicated successful perfusion. The fish were wrapped in wet paper toweling and allowed to fix for an additional hour.

For scanning electron microscopy, ovarian and egg samples were dissected from perfused fish and stored in fixative solution for a further 30 min to 1 h, then rinsed in two changes of rinsing buffer for 10 min each (2.14 g sodium cacodylate dissolved in 90 mL of distilled water with the pH adjusted to 7.4 with 0.1 N HCl and the osmolarity adjusted to 440 mOs with NaCl (about 1.6 g)). The rinsing buffer was replaced with standard osmium tetroxide fixative for 1 h. The samples were rinsed in two changes of rinsing buffer and dehydrated in a graded series of ethanols. Ethanol was replaced with amyl acetate by passing the samples through a graded series. The samples were dried in a critical point apparatus using CO_2 replacement of amyl acetate. Mounted eggs and ovarian tissue were gold coated and viewed in an ETEC Autoscan instrument using a 20 kV accelerating potential. Exposures were made on Ilford FP4 120 roll film, developed in Kodak HC 110 developer and printed on Ilford variable contrast paper.

For transmission microscopy, samples were dissected from perfused fish, rinsed in buffer, post-fixed in osmium tetroxide, rinsed twice again, and dehydrated in graded ethanols. Ethanol in late vitellogenic and ovulating eggs was replaced with propylene oxide in three changes. They were infiltrated for 1 h each in graded mixtures of propylene oxide in Spurr (1969) medium hardness epoxy embedding resin and allowed to infiltrate overnight. Ovulated and fertilized eggs were similarly treated, except that epoxy infiltration times were extended, and Spurr's hard formulation was used.

Epoxy resin infiltration of late vitellogenic and ovulating eggs occurred as expected, but infiltration of ovulated and fertilized eggs did not. Propylene oxide diffused readily from the latter, but the resin infiltrated less readily. During resin infiltration, ovulated eggs invariably shrank, became distorted, and popped audibly. Popped eggs assumed their original shape and floated. Longer residency times in each graded mixture did not result in satisfactory infiltration. Reasonable infiltration was achieved by puncturing fixed eggs prior to infiltration. The egg membrane and internal egg morphology were disturbed near the puncture, but satisfactory sections were obtainable opposite it.

Cured and trimmed blocks were 'silver' sectioned (65–85 nm) with glass knives, stained with alkaline lead citrate stain, and formulated as follows. Lead citrate (1 g) was suspended in distilled water to make 100 mL. Ten mL of 0.1 N NaOH was mixed with 10 mL of the suspension in a CO_2 free environment to make the final stain. Sections were stained for 10 min, and observed in a Phillips EM 300 transmission electron microscope with the accelerating potential at 80 kV.

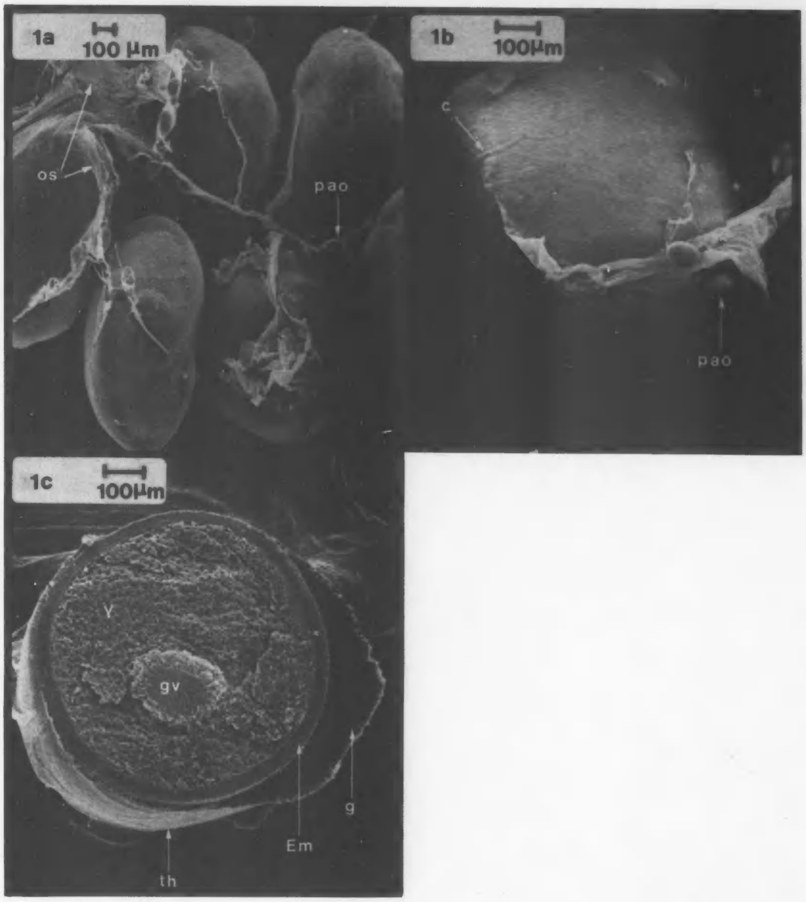

FIG. 1. Scanning electron micrographs of late vitellogenic oocytes. (1a) Several oocytes are shown. All have intact theca and granulosa layers. Abundant ovarian stroma (os) and 'pre-ovulatory atretic oocytes' (pao) are visible. (1b) A vitellogenic oocyte is shown with its associated stroma and 'pre-ovulatory atretic oocytes.' A capillary network (c) is evident beneath the thecal layer. (1c) A sectioned vitellogenic oocyte is shown. The germinal vesicle (gv), yolk (y), egg membrane (Em), granulosa cells (g), and thecal layer (th) are visible.

In order to verify the effectiveness of the perfusion fixation technique, several soft tissue types were dissected, minced under rinsing buffer, then similarly embedded and sectioned. Ultrastructural examination of kidney, optic nerve, retina, white and dark skeletal muscle, and intestinal tissue showed that the vascular perfusion method yielded excellent microstructural preservation. The perfusate did not initiate blood clotting.

Exposures were made on Kodak type 4489 electron microscope film, developed in Kodak D-19 developer and printed on Ilford variable contrast paper.

Results

Late vitellogenic oocytes were enclosed with follicles which were attached to ovarian stroma (Fig. 1a). Oocyte vascularization was discerned by raised ridges on the surface of the follicle (Fig. 1b). The follicle was comprised of two layers, the outer theca and the inner granulosa layer (Fig. 1c).

A class of small, non-vitellogenic oocytes (tentatively identified as pre-ovulatory atretic oocytes, or PAOs) was observed in late vitellogenic ovaries attached to ovarian stroma (Fig. 1a, 1b, 2a). At this stage, PAOs were covered with thecal, but not granulosa cells (Fig. 2b). PAO thecal tissue was absent in ovulating ovaries (Fig. 2c). PAOs were not seen in ovulated ovaries, nor did they accompany fertile eggs expressed from the urogenital papilla. They were seen in fixatives and other solutions, along with other ovarian debris, when eggs were dissected from ovulating ovaries. The PAO membrane consisted of two layers that appeared similar in structure and staining characteristics (alkaline phosphatase method with eosin counterstain; periodic acid Schiff stain for mucosaccharide with fast green counterstain, Gillis et al. 1990), to the internus and sub-internus of vitellogenic eggs.

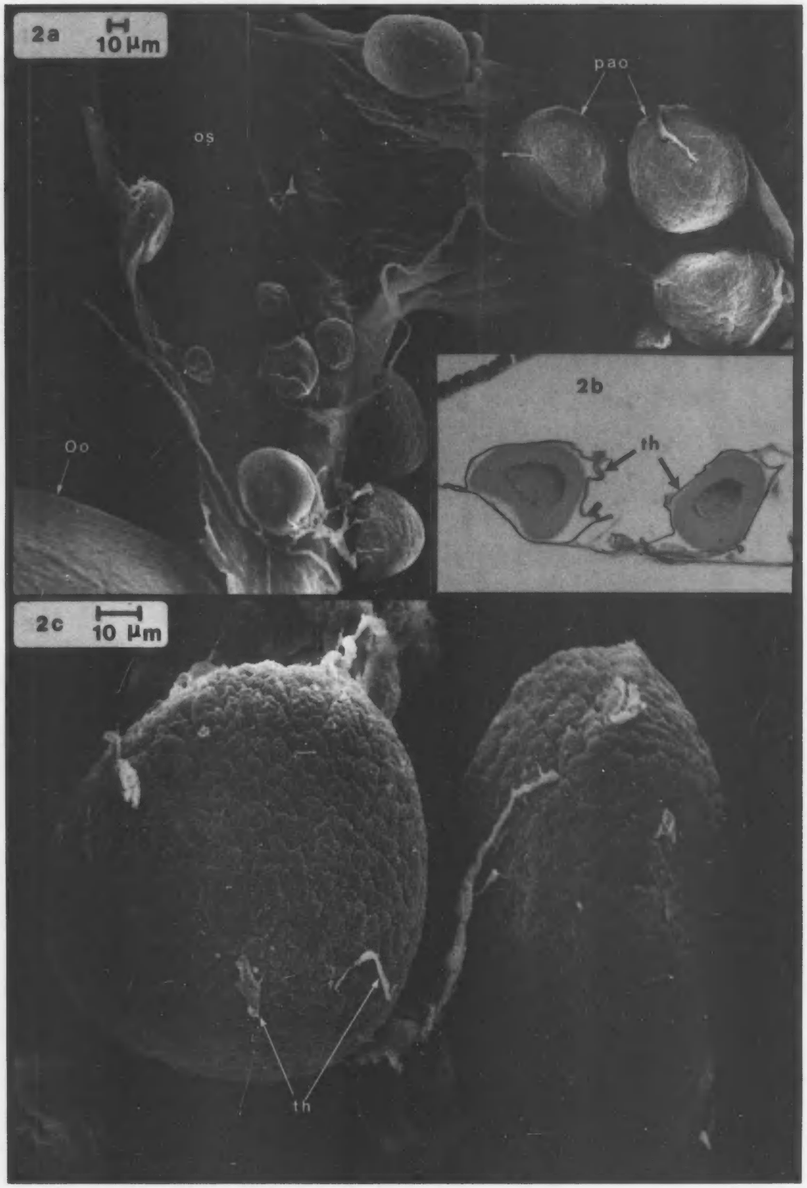

FIG. 2. Scanning electron micrographs of atretic oocytes from a late vitellogenic ovary. (2a) This view shows several 'pre-ovulatory atretic oocytes' (pao) and their association with the ovarian stroma (os). The atretic oocytes vary in size from 20 to 90 μM in this field. The external surface visible here is the thecal cell layer. The theca of a single vitellogenic oocyte (Oo) is visible in the lower right corner. (2b) Paraffin sections for light microscopy revealed that these oocytes are covered with thecal (th) cells but not granulosa cells. (2c) Two 'pre-ovulatory atretic oocytes' sampled from an ovulating ovary are shown. The surface of the structures is the internus, with small remnants of thecal tissue (th) attached.

Several structural changes accompanied ovulation. The definitive event was the expulsion of the oocyte from the follicle. Rents and tears appeared in the follicular and stromal tissue (Fig. 3a) and the oocytes were expelled from the follicle (Fig. 3b). The margin of the follicle constricted the emerging oocyte. The surface texture of the emerging oocyte was indented. Each indentation, formerly occupied by a single granulosa cell, contained numerous irregularly distributed pores

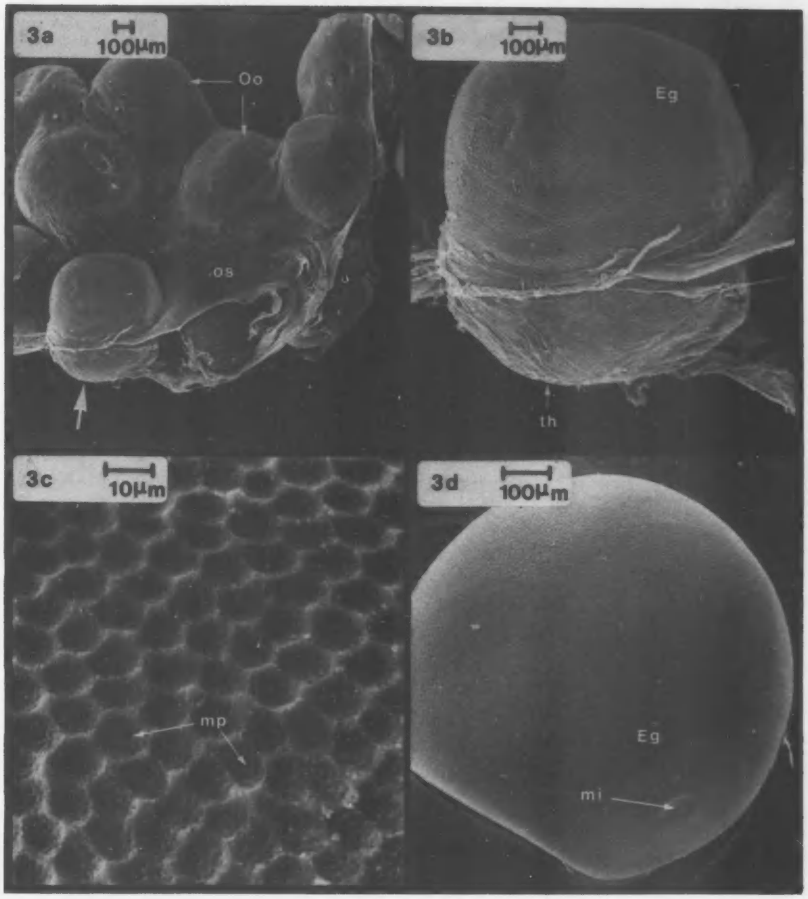

FIG. 3. Scanning electron micrographs of ovulating oocytes. 3a. Several oocytes (Oo) are shown enclosed within folds of ovarian stroma (os). Several rents are visible in the stroma and thecal layers. The egg identified by the heavy arrow has partly emerged from the degenerating membranous tissue. 3b. The emerging egg (Eg) identified in 2a is enlarged in this view. The oocyte appears constricted by the thecal tissue (th) in the equatorial region. 3c. This view shows an enlargement of the exterior surface of the externus. Numerous depressions are apparent. Each depression was formally occupied by a single granulosa cell. Membrane pores (mp) are irregularly distributed within each depression. 3d. The egg shown (Eg) is fully ovulated and has completed its first, or ovulation hydration (Gillis et al. 1990). The egg has taken on a more spherical form. The position of the micropyle (mi) is shown within a shallow crater. The lighter, circular area surrounding the micropyle crater may signify the absence of an adhesive epi-layer.

(Fig. 3c). The first or ovulation hydration accompanied ovulation (Gillis et al. 1990). The first hydration expanded the egg and smoothed much of its external texture. The egg assumed a more spherical appearance (Fig. 3d). Fertilized eggs swelled significantly in seawater, further smoothing both external and internal membrane texture (Fig. 4a). Eggs became strongly adhesive. Excess sperm adhered to the surface of the egg (Fig. 4b).

The membrane of Pacific herring late vitellogenic oocytes consisted of five layers (Fig. 5a). The sub-internus was located next to the oocytes plasma membrane and was laminar in structure. The laminations were compacted when compared with those of the internus. The structure of the sub-internus did not change appreciably during or after ovulation (Figs. 5b and 6a). The sub-internus was separated from the internus by a thin layer of loosely packed, fibrous material described as the proximal inter-layer (Fig. 7b). The internus was observed external to the proximal inter-layer (Fig. 5a). The internus was similar in structure to the sub-internus except that the former was slightly thicker and the laminations were more widely-spaced and fewer in number. The average number of laminations in the internus increased from seven to eight, from late vitellogenic to ovulated eggs while those in the sub-internus increased from 17 to 19.

Pores in the sub-internus ran relatively straight, taking the

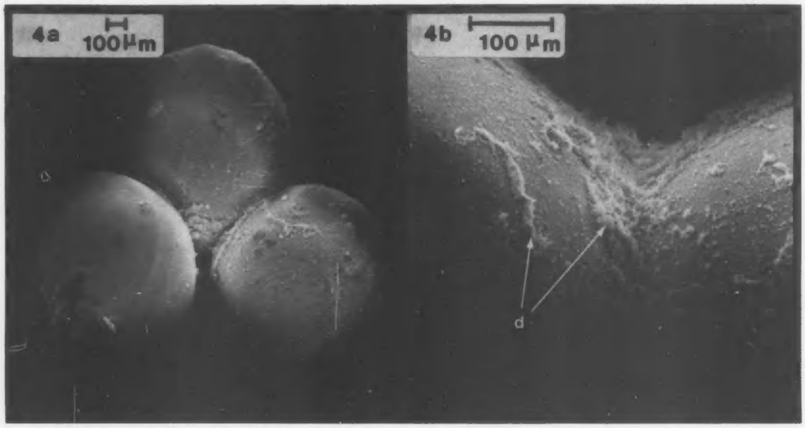

FIG. 4. Scanning electron micrographs of fertilized, water-hardened eggs. 4a. Three adherent eggs are shown in this view. 4b. An enlargement of the point between two adherent eggs is shown. Debris (d) (excess sperm) is shown adhering to the exteror surface of the externus. There is a particularly heavy accumulation near the joint. These eggs have completed their second hydration, and have swollen markedly. The egg surface is smooth and featureless.

shortest route from the ooplasm to the next layer. Microvilli originated from the oocyte and were observed in the pores. Pores in the internus took indirect, possibly helical paths (Fig. 5a) in late vitellogenic oocytes. By the time the egg had fully ovulated, however, the internus and sub-internus pore structure had collapsed. Internus pores were absent from ovulated and fertilized eggs (Fig. 6a, 6b).

An additional thin inter-layer was observed between the internus and externus (Fig. 5a, 5b). This layer (distal inter-layer) was approximately twice as thick as the proximal inter-layer and consisted of granular material (Fig. 7a). There were no changes observed in this layer during ovulation.

A fifth layer (externus) was the outer-most in late vitellogenic oocytes (Fig. 5a). Pores in this layer were straight and larger in diameter than those in either the internus or sub-internus. This layer was not laminated and appeared homogeneous in structure. The externus was unchanged in the ovulating oocyte sections but appeared spongy in sections of fully ovulated eggs (Fig. 6a). The spongy texture developed during the latter stages of ovulation but was not observed in sections of fertilized eggs (Fig. 6b).

During the early stages of ovulation, a sixth layer was deposited on the external surface of the egg (Fig. 5b, 6a). This epi-layer (Fig. 7c) appeared thin, compressed, and electron-dense in ovulated eggs; but expanded and irregular on eggs fertilized in seawater.

The terms 'sub-internus,' 'internus' and 'externus' appear elsewhere in the literaure (Groot and Alderdice 1985), and are applied to analogous structures in Pacific herring. The terms 'proximal inter-layer,' 'distal inter-layer,' and 'epi-layer' are used here in their descriptive context.

Discussion

The structure of the Pacific herring oocyte membrane differs from that reported elsewhere. Dumont and Brummet (1980) report nine strata in the egg membrane of *Fundulus heteroclitus*, but many of these appear to be similar to the laminations of the Pacific herring internus and sub-internus. Groot and Alderdice (1985) found three layers in salmonid eggs which appear analogous to the three thickest layers of herring egg membranes. For this reason, Groot and Alderdice's terminology (externus, internus, and sub-internus) have been applied to similar appearing structures in Pacific herring oocyte and egg membranes. The proximal and distal inter-layers do not appear to have been described elsewhere. Names for these layers should await some elucidation of their function(s).

The small, non-vitellogenic oocytes seen in late vitellogenic ovaries may constitute a class not previously described. Here, they are tentatively described as pre-ovulatory atretic oocytes, or PAOs. Pre-ovulatory atretic oocytes are smaller than, and dissimilar in structure to, atretic oocytes described in Hay et al. (1987). Pre-ovulatory atretic oocytes are similar in form, but somewhat smaller in size, than are Hay's (1987) previtellogenic oocytes. Bowers and Holliday (1961) describe late vitellogenic herring ovaries in which "... about one-tenth of the ovary showed signs of regression and in all instances the eggs had reached an advanced stage of development with the chorion fully formed and the presence of abundant yolk." Degeneration and absorption proceed "... until all that remains is a scar of connective tissue containing the shrunken and distorted chorion." These degenerated eggs do not appear to be analogous to PAOs. During ovulation, PAOs lost their covering of thecal tissue, became dissociated from the ovarian stroma, and were seen in the ovarian debris dissected from ovulating ovaries, yet were not seen in ovulated ovaries, or accompanying naturally spawned, mature eggs. Are PAOs degraded during ovulation and hence truly atretic, or are they resting oocytes fated to become vitellogenic in subsequent spawning seasons? We expect to ascertain the fate of PAOs in our ongoing work.

The epi-layer was deposited during the early stages of ovulation. Because the epi-layer was deposited during the breakdown of the granulosa cells (Fig. 5b), these cells were more

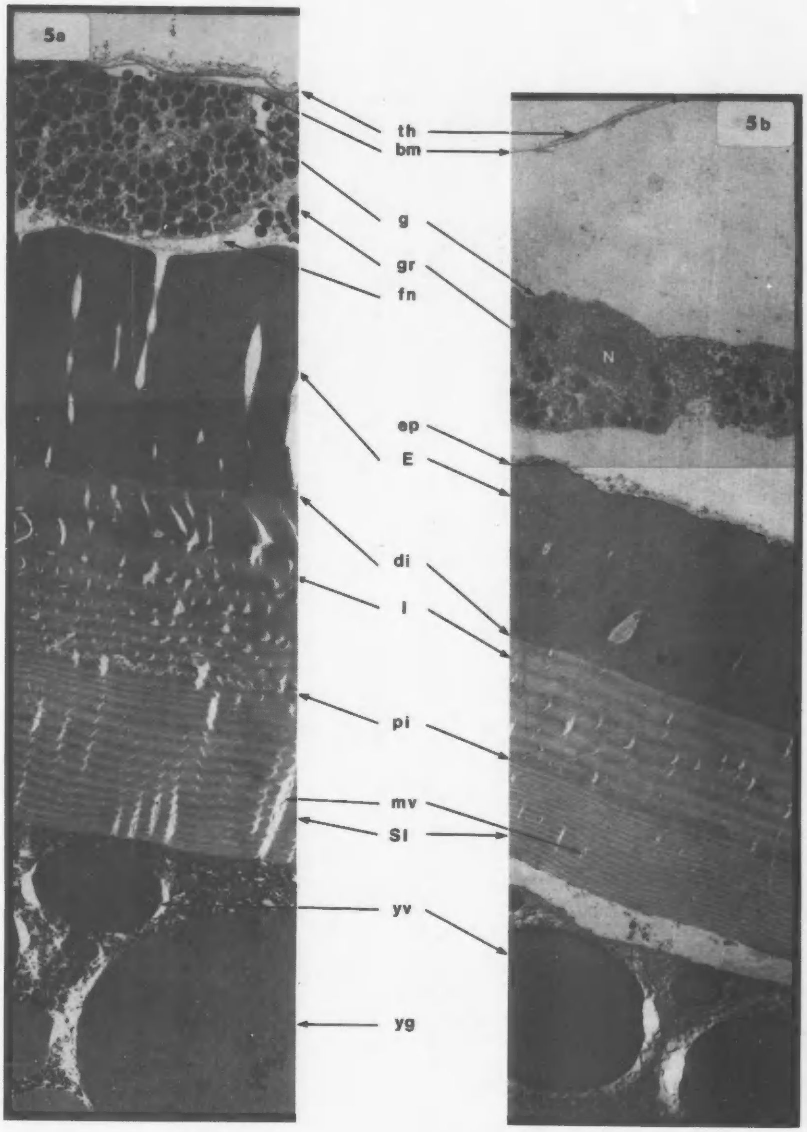

FIG. 5. Transmission electron micrographs of late vitellogenic and ovulating oocytes, (2400×). Sections of late vitellogenic and ovulating oocytes are shown in 5a and 5b, respectively. The observable structures are the thecal cells (th), basement membrane (bm), granulosa cells (g), granulosa cells granules (gr), fibrillar network (fn) in the vitellogenic egg, the epi-layer (ep) in the ovulating egg, externus (E), distal inter-layer (di), internus (I), proximal inter-layer (pi), microvilli (mv) within membrane pores, subinternus (SI), yolk vesicles (yv) and yolk globules (yg). Membrane pores are seen in all three membrane layers in both sections. In 5b, the epi-layer is visible on the external surface of the externus. The pores of the internus remain open. The space between the sub-internus and the yolk cytoplasm in 5b is an artifact.

likely responsible for epi-layer secretion than the club-shaped cells living the ovarian portion of the oviduct as was suggested by Bowers and Holiday (1961). Because of the location of the epi-layer, its structural change on contact with seawater, and the concurrent development of adhesion, we concluded that the expanded epi-layer constituted the adhesive substance.

The occurrence of fragile roes reported by sac-roe producers is likely associated with the failure of some important characteristic of the adhesive epi-layer. The cause of adhesion failure was not clearly explained by this study, however, some of the findings did suggest possible causes. The epi-layer was deposited on the external surface of the oocyte during early ovulation when the ovarian fluid also contained PAOs and other debris from the degradation of ovarian stroma and follicle cells. If harvested and processed in this condition, especially if the epi-layer was incompletely formed, then the debris could interfere with tight adhesion of adjacent eggs and lead to the 'fragile roe' condition. If this was the principal cause of 'fragile roe,' then the condition could be expected to be more frequent when fisheries are scheduled too early relative to the maturation of the fish. However, there may be other causes. For instance, although ovulation in Pacific herring is highly synchronized, a few females ovulate well in advance of spawning. In extreme cases, age-related changes occur in the epi-layer rendering it less adhesive. Accordingly, fragile roes may also occur in fully ovulated but aged ovaries. These two suggested sources of 'fragile roe' do not preclude other possible causes. For example, it is known that the 'fragile roe' condition can sometimes arise through inappropriate handling of the fish in the processing plants (Tsuyuki and Cheng 1985a, b).

The electron-dense epi-layer was similar to a thin layer identified in salmonid eggs by Groot and Alderdice (1985). They concluded that this layer caused the transition from a porous oocyte membrane to a semipermeable one. Their conclusion was supported by the appearance of annulated plugs in the external openings of the membrane pores. In Pacific herring eggs the thin epi-layer was not associated with plugs or other occluding mechanisms. Further, the pore structure of the herring egg externus remained open after ovulation. We concluded that the herring externus is not associated with semipermeability. We suggest that semipermeability was achieved during the first or ovulation hydration as follows: During vitellogenesis, the pores in the internus took indirect, possibly helical paths through the internus, as revealed by TEM. During the first hydration, the egg swelled, stretching and compressing the total egg membrane. The helical pores in the internus collapsed rendering the membrane semipermeable. This suggestion was supported by the following evidence. First, the pores of the internus were fully open in late vitellogenic and ovulating oocytes, but fully occluded in all ovulated eggs examined. Second, the onset of semipermeability during ovulation was indirectly demonstrated during infiltration with epoxy resin for TEM. Late vitellogenic and ovulating oocytes infiltrated readily, indicating that the pores of the oocyte membrane were still open. The relatively large epoxy molecules moved easily into the oocyte through the porous membrane as the transition solvent (propylene oxide) diffused out. By contrast, epoxy infiltration of ovulated eggs occurred very slowly, or not at all. While the relatively light transitional solvent still diffused easily from the egg, the large epoxy resin molecules diffused in less readily. The eggs shrivelled. Evidently, the pressure inside the egg was

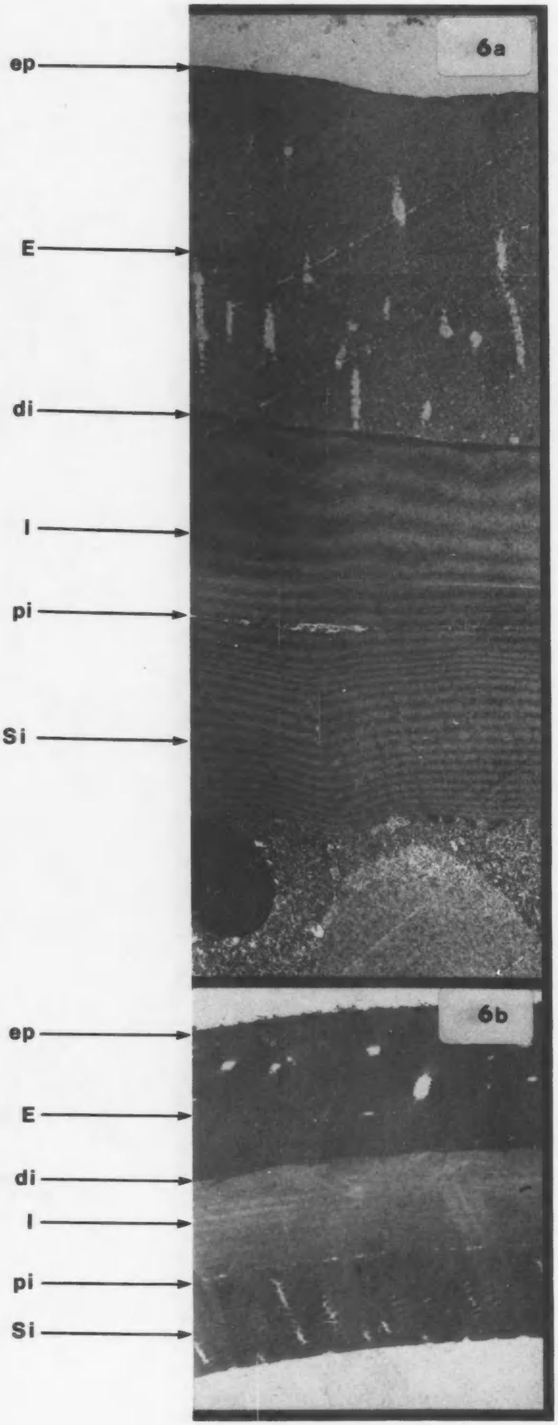

FIG. 6. Transmission elecron micrographs of ovulated and fertilized, water-hardened eggs (2900×). Sections of ovulated and fertilized, water-hardened eggs are shown in 6a and 6b, respectively. Observable structures include the epi-layer (ep), externus (E), distal inter-layer (di), internus (I), proximal inter-layer (pi) and subinternus (SI). The externus in the ovulated egg has taken on a 'spongy' appearance. Membrane pores are visible in the externus and sub-internus of both sections. Note, however, that the pores are not observable in the internus in either section.

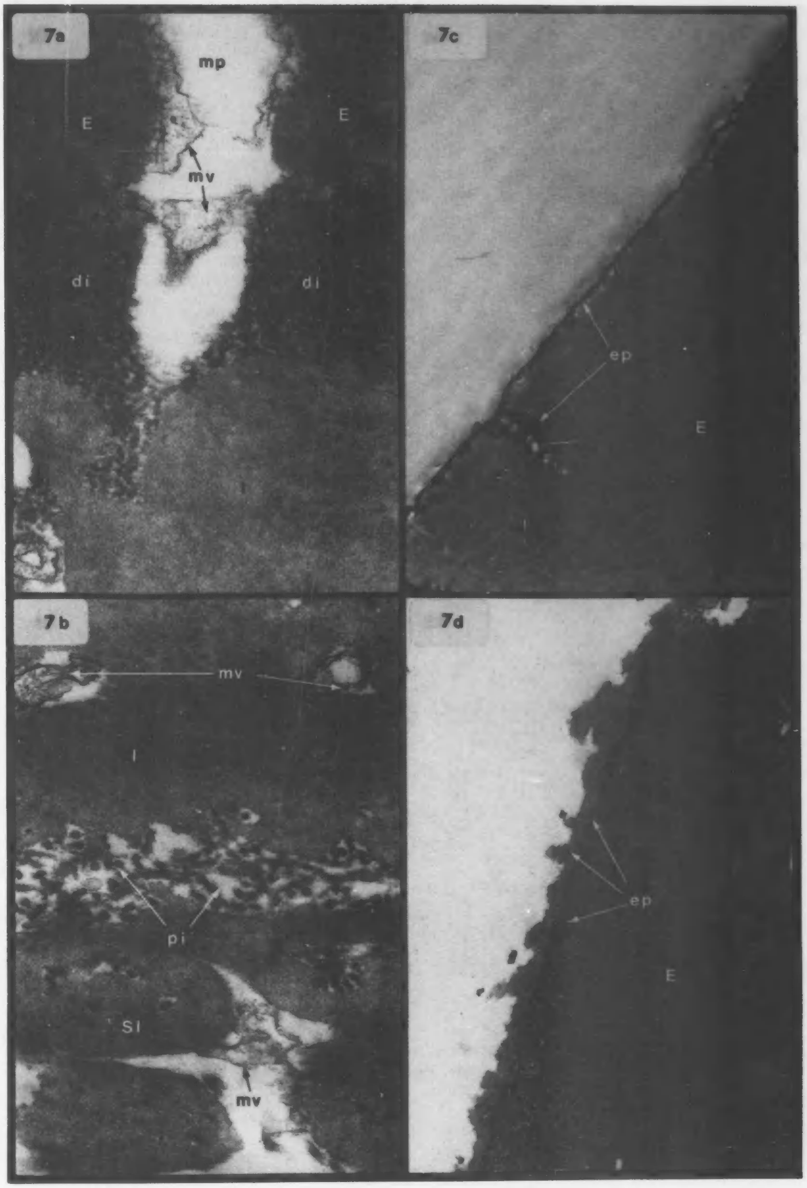

FIG. 7. Transmission electron micrographs of proximal and distal inter-layers and the epi-layers. 7a. The distal inter-layer (di) consists of numerous small granules. Also visible are the externus (E), internus (I), and remnants of microvilli (mv) in an egg membrane pore (mp) (36 000 ×). 7b. The proximal inter-layer (pi) is shown in high magnification to consist of fibrous material. Also shown are the internus, sub-internus and microvilli (36 000 ×). 7c. This is a magnified view of the surface of the externus (E) showing the epi-layer (ep) responsible for egg adhesion in an ovulated egg (11 100 ×). Shown is the epi-layer of a fertilized, water-hardened egg that has been activated, rendering the egg strongly adhesive (16 800 ×).

so reduced that the vapor pressure of the transition solvent was reached resulting in a violent 'pop.' If the epi-layer, deposited during ovulation, caused egg semipermeability, then slow epoxy infiltration would have been encountered in ovulating as well as ovulated eggs. While the collapse of the internus pore structure appears to be the most likely cause of egg membrane semipermeabiilty, other possible causes ae not precluded.

References

Bowers, A. B., and F. G. T. Holliday. 1961. Histological changes in the gonad associated with the reproductive cycle of the herring (*Clupea harengus L.*) Mar. Res. 5: 3–16.

Dumont, J. N., and A. R. Brummet. 1980. The vitelline envelope, chorion, and micropyle of *Fundulus heteroclitus* eggs. Gamete Res. 3: 25–44.

Gillis, D. J., B. A. McKeown, and D. E. Hay. 1990. Physiological and histological aspects of late oocyte provisioning, ovulation and fertilization in Pacific herring (*Clupea harengus pallasi*). Can. J. Fish. Aquat. Sci. 47: 1505–1512.

Groot, E. P. and D. F. Alderdice. 1985. Fine structure of the external egg membrane of five species of Pacific salmon and steelhead trout. Can. J. Zool. 63: 552–566.

Hay, D. E., D. N. Outram, B. A. McKeown, and M. Hurlburt. 1987. Ovarian development and oocyte diameter as maturation criteria in Pacific herring (*Clupea harengus pallasi*). Can. J. Fish. Aquat. Sci. 44: 1496–1502.

Hinton, D. E. 1975. Perfusion fixation of whole fish for electron microscopy. J. Fish. Res. Board Can. 32: 416–422.

Hjort, J. 1910. Report on herring investigations until January, 1910. Publ. Circ. Cons. Explor. Mer No. 53.

Rosenthall, H., and P. Odense. 1986. Fertilization in herring eggs: a scanning electron microscope study. Int. Counc. Explor. Sea H: 40 6 p.

Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Ultrastruct. Res. 26: 31–43.

Tsuyuki, H., and J. Y. H. Cheng. 1985a. Quality and yield of roe from impounded herring. Can. Tech. Rep. Fish. Aquat. Sci. 1408: 25 p.

1985b. The effect of maturity, handling and processing conditions, freezing methods on the development of fragile roe and on roe yield from impounded and wild stocks, 1984. Can. Tech. Rep. Fish. Aquat. Sci. 1411: 29 p.

Physiological and Histological Aspects of Late Oocyte Provisioning, Ovulation, and Fertilization in Pacific Herring (*Clupea harengus pallasi*)

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Late vitellogenic, ovulating, and ovulated Pacific herring oocytes were examined histologically and histochemically in order to understand these fine-scale processes and to evaluate egg maturation timing test criteria for use in resource management. Late vitellogenic events were investigated using an alkali-labile protein phosphorus (ALPP) assay of serum, liver, and gonad tissue. Serum levels were constant in males but declined in females. Ovarian levels declined (110 to 15 mg/L) during ovulation. Hepato-somatic indices were constant in males but declined in females (1.6 to 0.9%) during ovulation. The abundance of electron-lucent hepatocytes in female livers compared with males, their synthetic and secretory structures, and their reversion during ovulation suggest these cells as vitellogenin synthetic locations. During population ovulation, the gonosomatic index for females and males increased from 15–27%, and 15–23% respectively. Eggs hydrated from 67–76% during ovulation, and secondarily to 83% after fertilization. Unfertilized eggs dehydrated to 74% water. Alkaline phosphatase was localized in follicular granulosa and thecal cells suggesting these as locations of vitellogenin dephosphorylation. The test criteria; hepato-somatic index, lucent hepatocyte ratios, gonadosomatic index, and egg moisture are problematic but ovarian ALPP determination appears promising.

On a effectué un examen histologique et histochimique d'ovocytes de hareng du Pacifique à la fin de la vitellogénèse, au moment de l'ovulation et après l'ovulation afin de comprendre ces processus et d'évaluer les critères de détermination de la maturité des oeufs destinés à la gestion de la ressource. On s'est servi d'une analyse de la teneur en phosphore alcali-labile (ALPP) des protéines du sérum, du foie et des gonades pour étudier les derniers tades de la vitellogénèse. Les teneurs dans le særum, constantes chez les mâles, ont diminué chez les femelles chez qui les teneurs dans les tissus ovariens ont aussi baissé (110 à 15 mg/l) au cours de l'ovulation. L'indice hépatosomatique, constant chez les mâles, est passé de 1,6 % à 0,9 % chez les femelles au cours de l'ovulation. L'abondance plus élevée d'hépatocytes transparents aux électrons dans le foie des femelles ainsi que leurs structures de synthèse et de sécrétion à rôle inversé au moment de l'ovulation portent à croire que ces cellules sont le site de synthèse de la vitelline. Au moment où l'ovulation s'est produite dans une population, l'index gonadosomatique des femelles et des mâles est passé respectivement de 15 à 27 % et de 15 à 23 %. L'hydratation des oeufs est passée de 67 % à 76 % au moment de l'ovulation et à 83 % après la fertilisation tandis que les oeufs non fertilisés se sont déshydratés à 74 %. La présence de phosphatase alcaline dans les cellules foliculaires et thécales porte à croire qu'elles sont le site de la déphosphorylation de la vitelline. Les critères de détermination de la maturité des oeufs, soit l'indice hépatosomatique, la proportion d'hépatocytes transparents aux électrons, l'indice gonadosomatique et la teneur en eau des oeufs sont douteux mais la détermination du ALPP promet.

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Pacific herring contribute to British Columbia's economy chiefly through roe fisheries. Fully ovulated roe is required. The ability to utilize these roe fisheries arises from group-synchronous roe maturation and mass-spawning of the population. To conduct the fisheries, managers currently rely on yield determination (the ratio of ovulated roe to total sample weight) derived from time series sampling of target stocks. Fishing must be conducted between the time when most of the fish in a stock have ovulated, but before the onset of mass spawning. A fishery may be declared within hours of the estimation of acceptable yield. Understanding the fine-scale events surrounding herring ovulation may facilitate the development of tests that allow an earlier determination of roe maturation timing, and hence, an expansion of fishing opportunities. In addition, more detailed knowledge of the latter stages of maturation, spawning, and fertilization afforded by this investigation could improve our understanding of Pacific herring reproductive biology, and hence our ability to manage and conserve the wild stocks and their habitat.

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This study examined some relevant histological changes and physiological processes that occur in the ovary, eggs, and liver during this period. Gonosomatic and hepato-somatic indices, and ovarian/egg water content were measured to assess the degree of population maturation and ovulation synchronization. The progress of late vitellogenesis was assessed by time series measurement of liver, blood, and ovarian alkali-labile phosphorus. Light microscopy was used to localize alkaline phosphatase activity. Transmission electron microscopy was used to examine liver tissues of vitellogenic and ovulated females. Parallel measurements and observations were made in male herring to provide a basis of comparison to a non-vitellogenic member of the same species over the same time interval.

Materials and Methods

Pacific herring were captured by purse-seine in the Gulf Islands, 20–30 km south of Nanaimo, British Columbia in January and early February of 1984. They were transported by tank barge, and impounded in marine net-pens at the Pacific Biological Station in Departure Bay, Nanaimo. Approximately 25 mt of herring (>150 000 individuals) were confined in a master impoundment.

Two subsamples of about 1 000 fish each were removed from the master impoundment, and housed in two small impoundments of about 30 m^3. The fish were allowed to adjust to their surroundings for 7 d prior to sampling. The fish were not fed throughout the impoundment and sampling period. Mortality related to fishing and impoundment was estimated at 5%. Subsequent mortality was estimated at less than 1% per week. The impoundment stocking density in the small impoundments was estimated at 1–2 kg/m^3. There was no evidence of predation during the study period.

According to data from previous years of test fishing, population ovulation was estimated to occur during the first 2 wk of March. Accordingly, sampling from the small impoundments was conducted on February 16 and 23; March 1, 5, 8, 9, 10, 12, 13, 14, 15, 16, 19, 22, and 29; and April 5 and 12. For each sample the impoundment web was raised to concentrate the fish near the surface. Forty to 60 fish were dipnetted randomly from the impoundment and transported in plastic pails containing seawater to the laboratory. Transport time was 7–10 min. Fish under 100 g, and injured or diseased fish were removed from the sample. Ten each of male and female fish were subsampled randomly from the remaining fish. For late vitellogenic and ovulating fish, gender was determined by making a small incision in the lateral body wall and examining the gonads directly. Gender of sexually mature fish was determined by the nature of the gametes that extruded spontaneously from the urogenital papilla. Each fish was towel-dried. Standard length and weight were measured. Blood was drained from the caudal vessels into 1.5 mL polypropylene centrifuge vials. Blood serum was separated by centrifugation and stored on dry ice. Gonads and livers, including gall bladders, were dissected from each fish and weighed. Ovarian subsamples and livers were stored on dry ice. All tissues, except serum, were sampled in duplicate. Hepato-somatic and gonosomatic indices were calculated from weights measured during sampling, as were the percent of the total somatic weight consisting of liver and gonad respectively.

Direct determination of alkali-labile phosphorylated protein (ALPP) has been verified by Emmersen and Petersen (1976) and Tinsley (1985), and was used in the present study because of the ease with which it could be modified to accommodate large numbers of samples of different tissues. The method was adapted from Martin and Doty (1949) and Wallace and Jared (1967) as follows. Samples of liver or gonad tissue (0.2–0.3 g) were weighed into 150 by 15 mm culture tubes and homogenized in glass distilled water to bring the volume to 5 mL. Serum samples (0.05–0.25 mL) were similarly diluted to 5 mL. Five mL of cold (1–3°C) 10% trichloroacetic acid (TCA) was added. The tubes were vortexed and held at 4°C overnight. The mixture was incubated at 90°C for 30 min, centrifuged at 3000 *g* for 10 min, and decanted. The precipitate was vortexed with 10 mL of ethanol, held at 90°C for 5 min, centrifuged at 3000 *g*, and decanted, followed by the same treatment with mixed solvents (ethanol:ether:acetone:chloroform; 2:2:2:1) without heating. The precipitate was then dissolved and the alkali-labile phosphorus liberated by incubation at 100°C for 15 min in 1 mL 2N NaOH. The solution was cooled in an ice bath and neutralized by adding 1 mL of 2N HCl. Five mL of an equal volume mixture of isobutyl alcohol and toluene was added, followed by 2 mL of a freshly prepared equal volume mixture of molybdate and silico-tungstate (Martin and Doty 1949) reagents. Alkali-labile phosphorus was extracted to the organic solvent fraction by vigorously inverting each tube 15 times followed by centrifugation at 3000 *g* for 5 min. The remainder of this spectrophotometric determination followed Martin and Doty (1949). Prepared standards ranged from 1.1 to 36 mg/L phosphorus. Standards and reagent blanks were run with each set of samples. The method was tested on composite tissue samples prior to its application to study samples.

Late vitellogenic and ovulating ovarian samples for water determination were taken in triplicate from each of the ten subsampled females, then weighted. To obtain samples of fertilized eggs, ovulated eggs were removed surgically from ovaries, then immersed and agitated in sperm suspensions in seawater. Unfertilized, water-hardened egg samples were taken from the same ovaries as were fertilized ones and treated identically, except that there were no sperm suspended in the seawater. Fertilized and unfertilized eggs were stored in seawater for 2 h prior to towel drying and weighing. The samples were dried in an evacuated glass desiccator containing Drierite (anhydrous calcium sulfate) overnight, then reweighed, and the percent water calculated. Hydration water salinities were determined argentometrically (Strickland and Parsons 1965).

For alkaline phosphatase localization, 80% ethanol-fixed ovarian and egg samples were ethanol-dehydrated, infiltrated and embedded in paraffin (56°C) within 24 h of collection, then stored at 4°C. Chloroform was used as the transition vehicle betwen ethanol and paraffin. Ovulated eggs did not infiltrate as expected. The chloroform diffused freely from the egg but the paraffin infiltrated less readily, resulting in shrunken and deformed sections of ovulated eggs. Late vitellogenic and ovulating oocytes infiltrated normally. Trimmed tissue blocks were soaked in distilled water overnight to facilitate 10 μM sectioning. Block softening did not interfere with alkaline phosphatase reactions. A modified Gomori method was used as follows. Sections were deparaffinized in chloroform and hydrated in graded ethanol solutions followed by a 45 min incubation at 37°C in a solution consisting of 30 mL of solution A, (1.2 g $CaCl_2$; 0.5 g $MgSO_4 \cdot 7H_20$; 6.1 g hydroxymethyaminomethane; distilled water to make 1 L) and 50 mL of solution B (1% solution of sodium beta-glycerophosphate) with the pH adjusted to

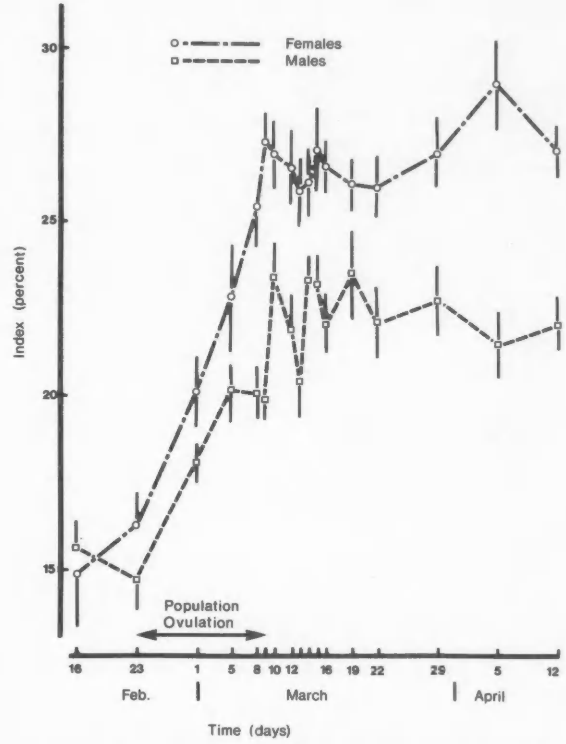

FIG. 1. Gonosomatic indices for male and female Pacific herring. The gonosomatic index is the percentage of total weight (including gonads) comprised of gonad weight. Each plotted point represents the mean for 10 fish. The vertical bars are standard errors of the mean values.

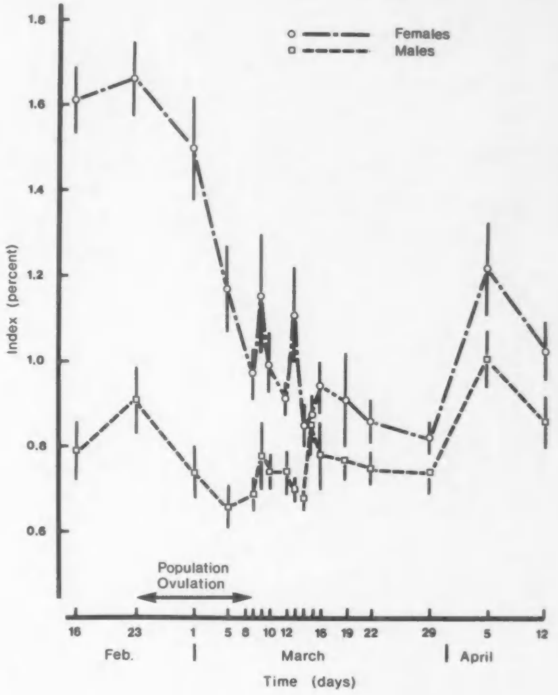

FIG. 2. Hepato-somatic indices for male and female Pacific herring. The hepato-somatic index is the percentage of total weight (including gonads) that is comprised of liver tissue. Liver weights included the weight of the gall bladder which was invariably full. Each plotted point represents the mean for 10 fish. The vertical bars are standard errors of the mean values.

9.8 with 0.1N HCl. The sections were then washed in distilled water, immersed in 2% cobalt nitrate for 5 min, washed in distilled water, immersed in 2% yellow ammonium sulfide for 2 min, washed in distilled water, counter-stained with eosin, dehydrated in graded ethanols, cleared in xylene, and mounted. Control sections were dry-heated to 100°C prior to processing to destroy enzyme activity. LM sections were viewed and photographed using a photomicroscope. Monochrome and colour exposures were made on Kodak 35 mm type 5060 Panatomic-X film developed in Kodak HC-110 developer, and type 5018 Ektachrome film with E-6 processing respectively. Liver tissue was prepared for transmission electron microscopy, viewed, and photographed according to Gills et al. (1990).

Correlation, ANOVA, and Student's *t*-test were applied to the data (Zar 1974). Conventional algorithms were used (Scalzo and Hughes 1975; Coulombe 1983). Ratio data were arcsine transformed where appropriate.

Results

The gonosomatic index (GSI) for females rose from 15% at the beginning of the study period to approximately 27% after ovulation (Fig. 1). The GSI for males rose from 15–23% during the same period. For males and females, the pre-ovulatory rise in GSI was significant. The postovulatory variability in GSI values for both males and females was not significant. The mean GSI values for males were always lower than those for females except for the first sampling day. Within the sampling period (February 23–April 12) a shorter period called 'population ovulation' is shown from February 23–March 9. During this time there was always one or more females that was fully ovulated with ripe eggs flowing freely in the lumen of the ovary. By March 9 all of the females sampled were fully ovulated.

At the beginning of the sample period female mean hepatosomatic index (HSI) values were twice as high as those for males (Fig. 2), and remained higher. Mean HSI values for males remained constant over the sampling period. There was no statistical significance in the variability of male values. The decline in values for females over the pre-ovulatory period (February 16–March 9) was significant but the variability following ovulation up to March 29 was not.

A test of the modified ALPP method using composite tissue samples confirmed the accuracy of the method ($n=10$; range, 37.4–42.8 mg/L; SD 1.4 mg/L; mean, 40.0 mg/L; SE 0.47 mg/L). Precision was confirmed by analyzing prepared standards. In the study results, the apparent late vitellogenic increase in female gonad ALPP was not statistically significant but the decline during ovulation was (Fig. 3). The declines in values for female serum and liver over the total sample period were both significant. During late vitellogenic and ovulation

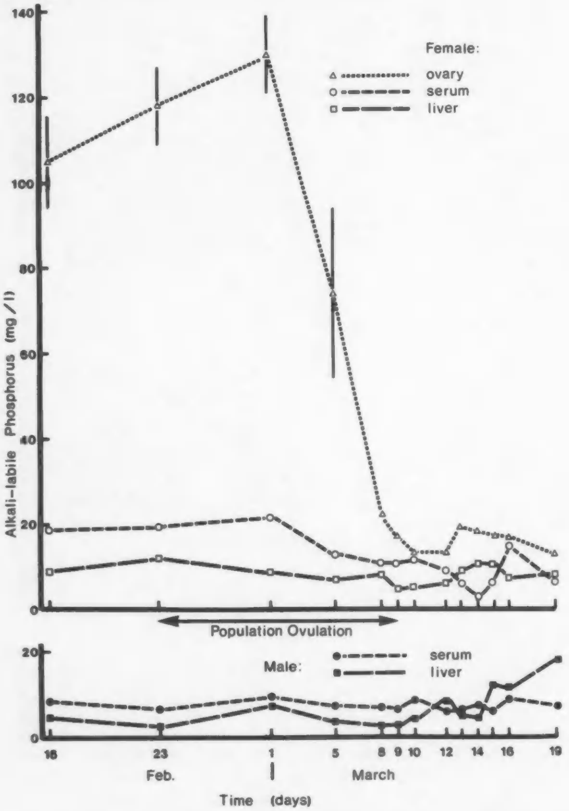

FIG. 3. Alkali-labile protein phosphorus for serum and liver in males and females, and ovaries in female Pacific herring. Each plotted point represents the mean value for 10 fish. The vertical bars represent standard errors of mean values.

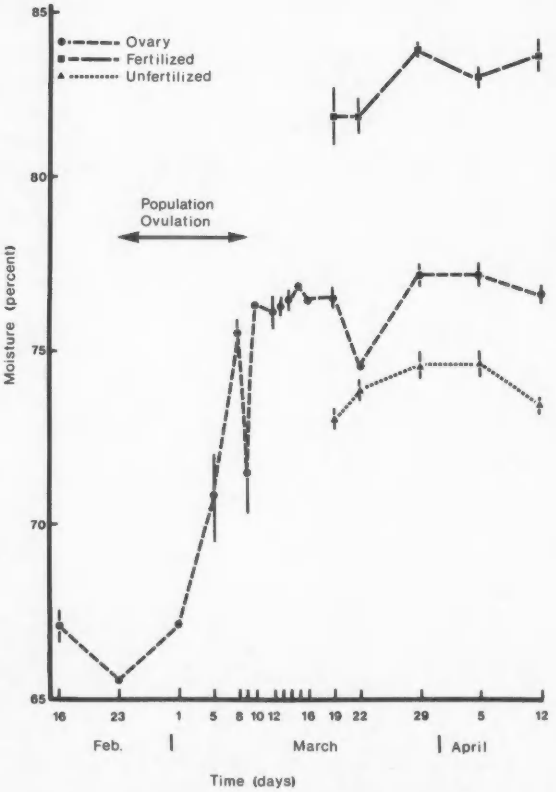

FIG. 4. Percent water content for ovaries, and fertilized and unfertilized water-hardened eggs of Pacific herring. Each plotted point represents the mean for 10 fish. The value for each fish is the mean for triplicate determinations. Each point therefore represents 30 determinations.

periods mean values for female serum were higher than those for female livers. Mean values for female liver were higher than those for male liver.

Herring eggs underwent two distinct hydrations (Fig. 4), the first occurring during ovulation. Average ovarian/egg water increased from 66–76% and remained at the latter level until the eggs were fertilized. During hardening in seawater following fertilization, the eggs hydrated to approximately 83% water. Spawned, unfertilized, water-hardened eggs dehydrated to 74% water. The difference between ovulated, fertilized, water-hardened and unfertilized, water-hardened egg/ovarian water was statistically significant. The amount of water taken up during the second hydration was not correlated with the salinity of the hydration water ($r = 0.3$; $P > 0.05$) over the limited range of salinities tested (23.00–28.29 ‰). The amount of water lost by unfertilized eggs was likewise not correlated with salinity ($r = 0.41$; $P > 0.05$).

Alkaline phosphatase activity was localized in granulosa and thecal cells (Fig. 5). Staining was particularly heavy in the perimeter of the granulosa cells where they contacted the externus of the egg membrane. Granulosa cell granules and the thecal cells were particularly heavily stained. Oocyte microvilli and other oocyte structures did not stain. Alkaline phosphatase activity was not found in sections of ovulated eggs.

Liver tissue from both male and female herring contained both electron-dense (Fig. 6a) and electron-lucent hepatocytes (Fig. 6b, 6c, 6d, 6e). However, lucent hepatocytes were scarce in male livers and dominated those of females. Lucent hepatocytes from late vitellogenic females contained large, well-developed vacuoles, well-developed, dilated, rough, endoplasmic reticulum (RER), and Golgi structures closely associated with vacuoles (Fig. 6b, 6e). Those from ovulating females contained shrinking or small vacuoles, and compressed, but still distinct RER (Fig. 6c). Those from ovulated females contained very few, fragmentary vacuoles and less differentiated RER (Fig. 6d).

Discussion

The duration of a large spawning wave may be about 5–6 d or shorter (Ware and Tanasichuk 1989) but the duration of the period of ovulation may be longer (15 d in this study). In effect, the duration of the ovulation period in a Pacific herring population is a reflection of the degree to which the maturation rates

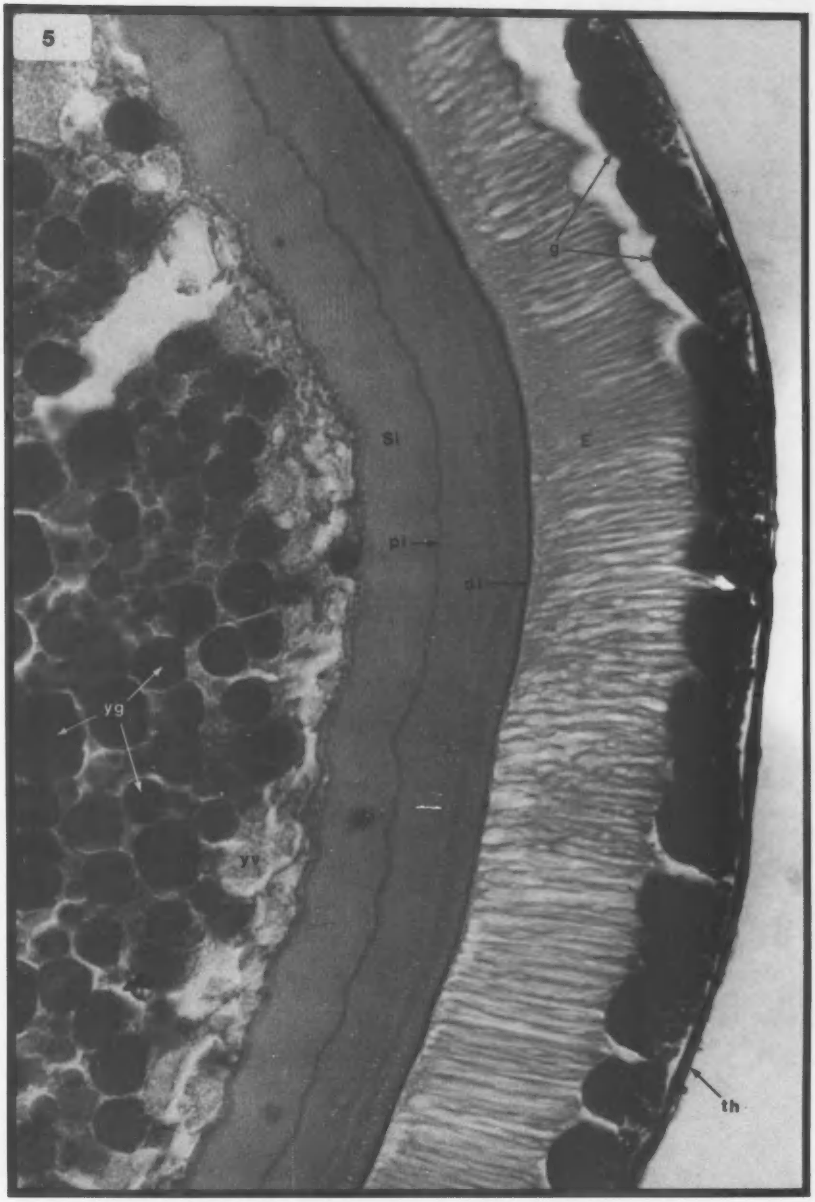

FIG. 5. Alkaline phosphatase localization — magnified view (Modified Gomori and eosin). This section of a late vitellogenic oocyte shows alkaline phosphatase activity in the granulosa (g) and thecal (th) cells. Enzyme activity is absent from oocyte microvilli located within the egg membrane pores. Also apparent in this section are the three principal layers of the egg membrane, the externus (E), internus (I), and sub-internus (Si), as well as two additional layers, the proximal inter-layer (pi) and distal inter-layer (di). Yolk vesicles (yv) and yolk globules (yg) are also visible (1100 ×).

differ among individuals. With a 15 d period of 'population' ovulation, it is essential that the earliest maturing herring have the capability of retaining viable, ovulated eggs within the ovary while waiting for the remainder of the population to ovulate (Hay 1986). The significance of this for management is that the time of spawning, and hence the optimal time for fisheries, depends on the variation in the degree of maturation within the population. To develop management tools, or tests for determining the degree of population maturation, the process itself must be clearly understood.

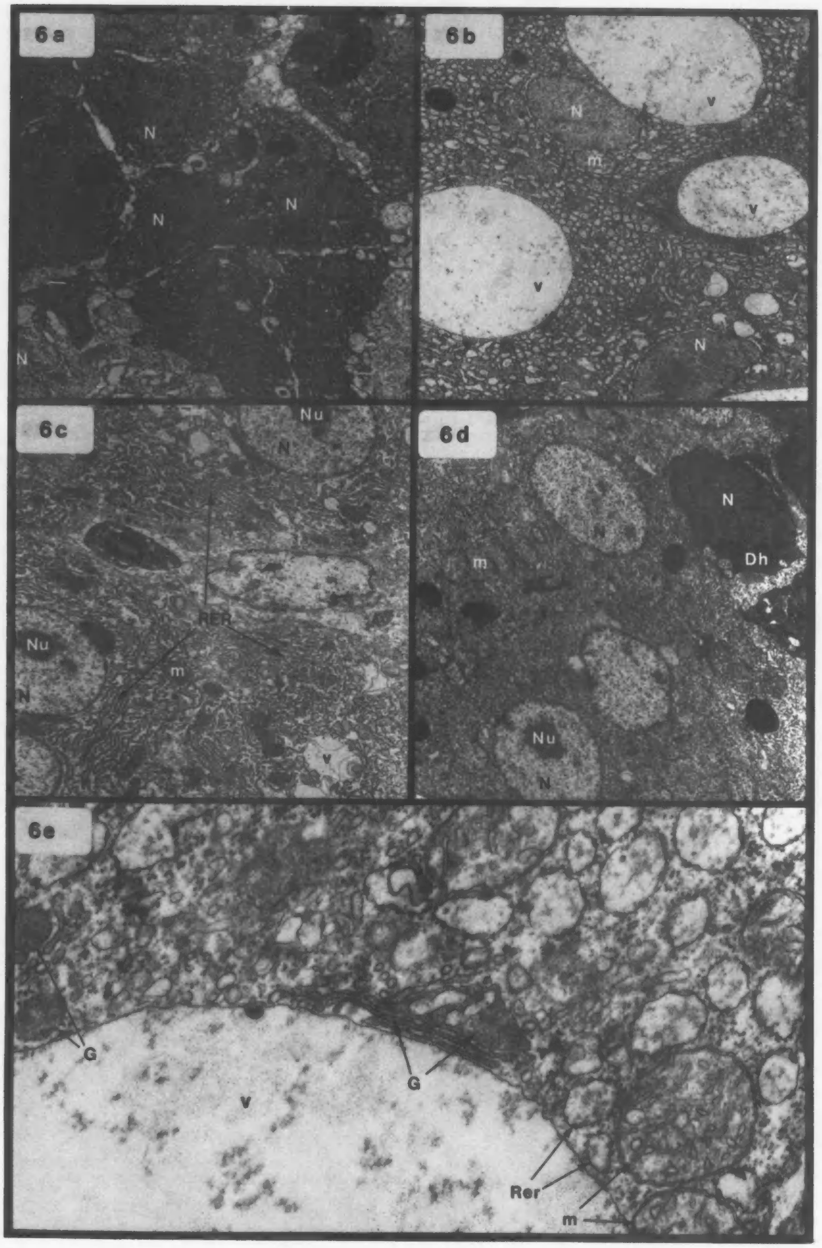

FIG. 6. Transmission electron micrographs of electron-dense and electron-lucent hepatocytes. Electron-dense hepatocytes are shown in 6a. Nuclei (N) and mitochondria are also identified in this section. Sections 6b, 6c, and 6d show sections of electron-lucent hepatocytes from late vitellogenic, ovulating, and ovulated females respectively (3 800 ×). Nuclei (N), nucleoli (Nu), rough endoplasmic reticulum (RER), secretory vacuoles (v), and mitochondria (m) are also identified. Figure 6e shows Golgi (G) associations in late vitellogenic oocytes, and tubular rough endoplasmic reticulum (Rer) with expanded lumens (32 000 ×).

Pacific herring feed heavily and accumulate energy in the spring and summer in offshore summer waters (Hay et al. 1987). Some of the energy is used during the early stages of gamete production but much is stored as lipid in the numerous fat bodies in the coelomic mesenteries. Most herring overwinter from December to February in deep waters near spawning beaches (Hay 1985). During this period, oocytes receive their final provisioning. There is very light feeding in winter, especially prior to final gamete maturation (Gillis 1979; Hay et al. 1988). Consequently, oocyte provisioning during late vitellogenesis must be derived from stored energy.

Most oocyte development consists of the accumulation of yolk material. Yolk vesicles, fated to become the cortical alveoli, appear first. Anderson (1968) demonstrated the association of vesicle formation and the internal synthetic structures of the oocyte and concluded that both glycoprotein synthesis and vesicle formation were endogenous. Lipid accumulation marks the commencement of endogenous vitellogenesis (Shackley and King 1977; Weigand 1982). Yolk globules, which expand to become the most abundant storage structure, form concurrently with, but after, the start of vesicle formation. Their endogenous origin from the fusion of numerous small coated vesicles has been confirmed by ultrastructural studies (e.g. Droller and Roth 1966; Anderson 1968; Shackley and King 1977). Exogenous vitellogenesis commences immediately after globule formation (Wallace and Selman 1981; Ng and Idler 1983).

Vitellogenin is an alkali-labile phosphorylated protein found in females and estrogenized males (Emmersen and Petersen 1976; Wallace 1978; Nath and Sundararaj 1981). Its occurrence in the blood plasma coincides with the appearance of yolk globules (Aida et al. 1973). The hormonal control of hepatic vitellogenesis has recently been elucidated by several authors (e.g. Wallace and Selman 1980, 1981; Kagawa et al. 1982; Nagahama et al. 1982; Nagahama 1983). Hepatocytes have previously been identified as the site of exogenous vitellogenesis, and the vascular system has been identified as the transferral route between the liver and the ovary (Wallace 1978; Sundararaj and Nath 1981; Ng and Idler 1983).

In this study, the hepatic source of vitellogenin for herring was suggested by the following evidence. The hepato-somatic index for females was higher than that for males, and declined significantly in females during population ovulation. In addition, ultrastructural changes in female hepatocytes were consistent with those described for vitellogenic zebrafish (*Brachydanio rerio*) (Peute et al. 1978) and rainbow trout (*Oncorhynchus mykiss*) (van Bohemen et al. 1981). The suggestion that electron-lucent hepatocytes were the sites of vitellogenin synthesis was supported by their abundance in female livers during late vitellogenesis, their abundant dilated RER, the presence of large secretory vacuoles with which well-developed and large Golgi bodies were associated, and the scarcity of lucent hepatocytes in male livers during all stages examined. (Golgi bodies have been shown to be the structure that concentrates and secretes vitellogenin in frog hepatocytes, Herbener et al. 1984). Secretory vacuoles were few, and appeared to be degenerating in lucent hepatocytes of ovulating females. Electron-lucent hepatocytes were less abundant in ovulated females and their internal structure more closely resembled that of electron-dense ones. These changes in lucent hepatocytes closely paralleled the decline in female hepato-somatic index that accompanied ovulation.

Although there is a clear relationship between the hepato-somatic index and ovulation, the use of the hepato-somatic index (Fig. 2) in a maturation test method has the disadvantage of utilizing liver weight which comprises a small fraction of somatic weight, thereby increasing the chance of experimental error during the weighing. Also, it is unknown if the hepato-somatic index is size dependent. Likewise, using the examination of livers for lucent hepatocytes is impractical due to time and apparatus requirements demanded.

Vitellogenin is comprised of a lipid-containing fraction, lipovitellin and a phosphorylated protein fraction, phosvitin. Mano and Lipman (1966) observed that there are several forms of phosvitin which differ in amount of phosphorylation as well as in other ways. At the start of exogenous vitellogenesis, non-phosphorylated phosvitin is incorporated by the oocytes. The degree of phosphorylation increases as vitellogenesis proceeds. The highest phosphorus levels occur prior to ovulation (Inoue et al. 1971; Mano 1969, 1970).

ALPP values for males (Fig. 3) provide a basis of comparison to values for females. Because males are not vitellogenic, ALPP values for males must arise from non-vitellogenin sources of phosphorylated protein. ALPP values measured in females that are greater than those for males likely result from vitellogenin. However, due to changes in the degree of vitellogenin phosphorylation during late vitellogenesis, values reported for females in Fig. 3 are considered to be relative, and not absolute.

Low ALPP levels in female liver compared with other tissues suggested that vitellogenin was secreted into the vascular system very quickly following its synthesis. Higher ALPP levels in female serum suggest that the sequestering mechanism was less efficient than the hepatocyte secretory mechanism, or alternatively that the vascular system acted as a store for vitellogenin thereby reducing possible synthetic inhibition that storing vitellogenin in hepatocytes may have generated.

Based on alkaline phosphatase localization results, the site of dephosphorylation of vitellogenin appears to have been the layer of follicular cells surrounding each oocyte. The following evidence supports this assumption. Alkaline phosphatase was localized in both thecal and granulosa cells. Staining was heaviest in the proximal apex of the granulosa cells and granulosa granules, suggesting that the apex of the cell may be the site of most vitellogenin dephosphorylation.

There is some evidence that vitellogenin rather than its cleavage products may be incorporated by oocytes (Selman and Wallace 1983). In Pacific herring, however, alkaline phosphatase activity in follicular cells, and widespread exocytotic activity from the bases of granulosa cells supported the suggestion that, at least during late vitellogenesis, vitellogenin was dephosphorylated within the follicular cells. As a quantitative measure, the level of ALPP in the ovary is promising because the pre-ovulatory increasing trend reverses, then declines, during the period of population ovulation (Fig. 3).

Female GSI increased more rapidly during late vitellogenesis than did that for males. late vitellogenesis may account for some of the weight increase. However, follicular cells detached, and began to degrade early in the process of ovulation, thereby precluding continued egg provisioning in individual fish (Gillis et al. 1990). Further, the ALPP levels evident during ovulation (Fig. 3) were likely related to variability in the 10-fish sample, where some of the fish in the sample would have been fully ovulated, while others would still be vitellogenic and contributing ALPP to the average results reported in the figure.

The first, or ovulation hydration, accounted for most of the increase in the GSI. This is supported by the observation that the increase in ovarian water closely paralleled increases in the gonosomatic index. The two were positively correlated during late vitellogenesis ($n = 170$; $r = 0.68$; $P < 0.01$). These findings suggest that during final maturation, vitellogenesis may be a consistent process, and the dramatic increase in GSI during ovulation, evident in this study and Hay et al. (1988) and others, results mostly from water uptake. This hydration at ovulation should not be confused with the second egg hydration at fertilization (water hardening). Hart and Yu (1980) showed that this hydration is caused by the high osmotic pressure associated with the release of colloids from the cortical alveoli immediately after fertilization. This process was demonstrated in the present study. Interestingly, unfertilized eggs dehydrated on exposure to seawater. Additional experimentation over a wider salinity range than that used in this study will be required to investigate possible relationships between salinity and the amounts of hydration or dehydration in fertilized and unfertilized eggs.

The gonosomatic index (GSI), while accurate and sensitive for assessment of an individual, may be only an approximation of the status of the whole population. Another aspect of the GSI as a maturation criterion is that it is positively related to total fish size (Hay et al. 1987; Ware and Tanasichuk 1989). The moisture content of the ovary or oocytes (Fig. 4) may be a very sensitive quantitative measure of ovulation but it may not be practical to use at sea because it would require the use of drying ovens and sensitive balances.

Based on the findings of this study, and other work, HSI, GSI and egg moisture may be unsuitable for use as maturation test criteria if used alone. Tests based on ALPP levels appear promising if used alone or in combination with other criteria. However, aside from this study, there are no other baseline data for ALPP concentrations in herring during late maturation and more work is required to establish this parameter as a useful quantitative criteron for monitoring late maturation.

References

Aida, K., P.-V., Ngan, and T. Hibiya. 1973. Physiological studies on gonadal maturation of fishes: I. Sexual differences in composition in plasma protein in Ayu in relation to gonadal maturation. Bull. Jpn. Soc. Sci. Fish. 39: 1091–1106.

Anderson, E. 1968. Cortical alveoli formation and vitellogenesis during oocyte differentiation in the pipefish, *Syngnathus fuscus*, and the killifish, *Fundulus heteroclitus*. J. Morph. 124: 23–60.

Coulombe, D. 1983. BASIC programs for advanced statistical analysis. University of Ottawa Press. Ottawa, Can. pp. 28-33, 42-50.

Droller, M. J., and T. F. Roth. 1966. An electron microscope study of yolk formation during oogenesis in *Lebistes reticulatus guppyi*. J. Cell. Biol. 28: 209–231.

Emmersen, B. K., and I. M. Petersen. 1976. Natural occurrence and experimental induction by estradiol 17-beta of a lipophosphoprotein (vitellogenin) in flounder (*Platichtys flesus*, L.). Comp. Biochem. Physiol. 54B: 443–446.

Gillis, D. J. 1979. Ponding and other factors affecting the quality of herring for the food industry. Fisheries and Oceans Industry Rep. 109.

Gillis, D. J., B. A. McKeown, and D. E. Hay. 1990. Ultrastructural observations on the ovaries and eggs, and the development of egg adhesion in Pacific herring, (*Clupea harengus pallasi*). Can. J. Fish. Aquat. Sci. 47: 1495–1504.

Hart, N. H., and S.-F. Yu. 1980. Cortical granule exocytosis and cell surface reorganization in eggs of *Brachydanio*. J. Exp. Zool. 213: 137–159.

Hay, D. E. 1985. Reproductive biology of Pacific herring (*Clupea harengus pallasi*). Can. J. Fish. Aquat. Sci. 42: 111–126.

1986. Effects of delayed spawning on viability of eggs and larvae of Pacific herring. Trans. Amer. Fish. Soc. 115: 155–161.

Hay, D. E., D. N. Outram, B. A. McKeown, and M. Hurlburt. 1987. Ovarian development and oocyte diameter as maturation criteria in Pacific herring (*Clupea harengus pallasi*). Can. J. Fish. Aquat. Sci. 44: 1181–1194.

Hay, D. E., J. R. Brett, E. Bilinski, E. M. Donaldson, D. T. Smith, G. A. Hunter, and A. Solmie. 1988. Experimental impoundments of prespawning Pacific herring: effects of feeding and density on maturation, growth and proximate analysis. Can. J. Fish. Aquat. Sci. 45: 388–398.

Herbener, G H., M. Bendayan, and R. C. Feldhoff. 1984. The intracellular pathway of vitellogenin secretion in the frog hepatocyte as revealed by protein A-gold immunocytochemistry. J. Histochem. Cytochem. 32: 697–704.

Inoue, S., T. Kaneda-Hayashi, H. Sugiyama, and T. Ando. 1971. Studies on phosphoproteins from fish eggs. I. Isolation and characterization of a phosphoprotein from the eggs of Pacific herring. J. Biochem. 69: 1003–1011.

Kagawa, H., G. Young, and Y. Nagahama. 19872. Estradiol 17-beta production in isolated amago salmon (*Oncorhynchus rhodurus*) ovarian follicles and its stimulation by gonadotropins. Gen. Comp. Endocrinol. 47: 361–365.

Mano, Y. 1969. Continuous splitting off of phosphate from phosvitins by phosphoprotein phosphatase. J. Biochem. (Tokyo) 66: 109–111.

1970. Mechanisms of phosphorylation of phosphoprotein during oogenesis in trout *in vivo*. Biochim. Biophys. Acta. 201: 284–294.

Mano, Y., and F. Lipman. 1966. Characteristics of phosphoprotein (phosvitins) from a variety of fish roes. J. Biol. Chem. 241: 3822–3833.

Martin, J. B., and D. M. Doty. 1949. Determination of inorganic phosphate. Anal. Chem. 21: 965–967.

Nagahama, Y. 1983. The functional morphology of teleost gonads p. 223–275. *In* W. S. Hoar, D. J. Randall, and E. M. Donaldson [ed.] 1983. Fish Physiology. Vol. IX.

Nagahama, Y., H. Kagawa, and G. Young. 1982. Cellular sources of steroids in teleost gonads. Can. J. Fish. Aquat. Sci. 39: 56–64.

Nath, P., and B. I. Sundararaj. 1981. Isolation and identification of female-specific serum lipophosphoprotein (vitellogenin) in the catfish, *Heteropneustes fossilis*. Gen. Comp. Endocrinol. 43: 184–190.

Ng, T. B., and D. R. Idler. 1983. Yolk formation and differentiation in teleost fishes, p. 373–404. *In* W. S. Hoar, D. J. Randall, and E. M. Donaldson, [ed.]. Fish Physiology. Vol. IX. Academic Press, New York, NY.

Peute, J., M. A. van der Gaag, and J. D. G. Lambert. 1978. Ultrastructure and lipid content of the liver of the zebrafish, *Brachydanio rerio*, related to vitellogenin synthesis. Cell Tissue Res. 186: 297–308.

Scalzo, F., and R. Hughes. 1975. Elementary computer assisted statistics. Manson/Charter Publishers Inc., New York, NY p. 246–261.

Selman, K., and R. A. Wallace. 1983. Oogenesis in *Fundulus heteroclitus*. III. Vitellogenesis. J. Exp. Zool. 226: 441–457.

Shackley, S. E., and P. E. King. 1977. Oogenesis in a marine teleost, *Blennius pholis* L. Cell Tissue Res. 181: 105–128.

Strickland, J. D. H., and T. R. Parsons. 1965. A manual of sea water analysis with special reference to more common micro-nutrients and to particulate organic material. Bull. Fish. Res. Board Can. 125: 203 p.

Sundararaj, B. I., and P. Nath. 1981. Steroid-induced synthesis of vitellogenin in the catfish, *Heteropneustes fossilis* (Bloch). Gen. Comp. Endocrinol. 43: 201–210.

Tinsley, D. 1985. A comparison of plasma levels of phosphoprotein, total protein and total calcium as indirect indices of exogenous vitellogenesis in the Crucian carp, *Carassius carassius* (L.). Comp. Biochem. Physiol. 80B: 913–916.

van Bohemen, C. G., J. G. D. Lambert, and J. Peute. 1981. Annual changes in plasma and liver in relation to vitellogenesis in the female rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 44: 94–107.

Wallace, R. A. 1978. Oocyte growth in non-mammalian vertebrates, p. 469–501. *In* R. E. Jones [ed.] The Vertebrate Ovary. Plenum, New York. NY.

Wallace, R. A., and D. W. Jared. 1967. Studies on amphibian yolk, VII. Serum phosphoprotein synthesis by vitellogenic females and estrone-treated males of *Xenopus laevis*. Can. J. Biochem. 46: 953–959.

Wallace, R. A., and K. Selman. 1980. Oogenesis in *Fundulus heteroclitus*. II. The transition from vitellogenesis into maturation. Gen. Comp. Endocrinol. 42: 345–354.

1981. Cellular and dynamic aspects of oocyte growth in teleosts. Am. Zool. 21: 325–343.

Ware, D. M., and R. W. Tanasichuk. 1989. Biological basis of maturation and spawning waves in Pacific herring (*Clupea harengus pallasi*). Can. J. Fish. Aquat. Sci. 46: 1776–1784.

Weigand, M. D. 1982. Vitellogenesis in fishes. *In* C. J. J. Richter, and H. J. Th. Goos [compilers] International symposium on reproductive physiology of fish proceedings. Wageningen Centre for Agricultural Documentation

Zar, J. G. 1974. Biostatistical analysis. Prentice—Hall, Englewood, NJ. p. 185–18; 220–211.

Stimulation of Plasma Thyroxine Levels by Novel Water Chemistry during Smoltification in Chinook Salmon (*Oncorhynchus tshawytscha*)

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The separate influences of changes in water chemistry and flow rate on plasma thyroxine levels were assessed in a freshwater population of chinook salmon (*Oncorhynchus tshawytscha*) undergoing smoltification. Fish subjected to a change in water source had plasma thyroxine levels significantly greater than those of controls on several sampling dates. Maximum levels were observed 10 d after water change. Conversely, plasma thyroxine levels in fish for which the flow rate was increased showed little change and were never significantly different from controls. Plasma thyroxine levels were greater in fish held under a natural rather than artificial photoperiod but the patterns of change were similar in the two groups. Control fish transported to a new hatchery and novel water source attained plasma thyroxine levels which were even greater than those of novel water-exposed fish at the original hatchery. The results indicated that altered water chemistry rather than increased flow rate was the stimulus for the elevation in plasma thyroxine levels associated with smoltification in these chinook salmon.

On a déterminé les influences individuelles des variations de caractéristiques chimiques et du débit de l'eau sur les niveaux de thyroxine sérique chez une population dulçaquicole de saumon quinnat (*Oncorhynchus tshawytscha*) en processus de smoltification. Les poissons soumis à une différente source d'eau ont montré des niveaux de thyroxine sérique nettement supérieurs à ceux des poissons témoins au moment de l'échantillonnage effectué à différentes dates. Les niveaux maximums ont été observés dix jours après le changement de la source d'eau. Inversement, les niveaux de thyroxine sérique chez les poissons gardés dans un milieu où le débit a augmenté n'ont que légèrement variés et n'étaient jamais nettement différents de ceux observés élevés chez les poissons soumis à une photopériode naturelle mais le régime de variation était semblable chez les deux groupes. Les poissons témoins transportés dans une autre écloserie et soumis à une nouvelle source d'eau ont vu leur niveau de thyroxine sérique atteindre un niveau plus élevé que celui observé chez les poissons gardés dans un milieu différent à la première écloserie. Les résultats révèlent que la variation des caractéristiques chimiques de l'eau et non l'augmentation du débit constitue le stimulus pour l'augmentation des niveaux de thyroxine sérique caractéristique de la smoltification chez les saumons quinnats.

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Timing of parr–smolt transformation (smoltification) in native populations of Pacific salmon is of interest to fisheries scientists, particularly in relation to the onset of seawater osmoregulatory ability and seaward migration. However, timing of smoltification is also of interest in the management of non-native, landlocked salmon stocks. In both cases one goal is to increase post-release survival and successful return of mature fish to the hatchery. In the midwestern United States chinook (*Oncorhynchus tshawytscha*) and coho (*O. kisutch*) salmon have been introduced as sport fish. Salmon are often raised in a hatchery in one geographic area and are transported for release into a lake or reservoir elsewhere. The attainment of seawater osmoregulatory ability is not essential for survival in freshwater populations. However, imprinting upon the appropriate hatchery facility or natal stream waters and migratory behavior of the smolt are two factors influenced by the timing of smoltification, and both are important to successful, self-supporting salmon introductions.

Smoltification is commonly monitored by three indices: ability to withstand a saltwater challenge, increase in gill Na^+–K^+ ATP-ase activity and plasma concentration of thyroxine (Dickhoff et al. 1982b). Thyroxine is thought to play a role in several aspects of smoltification including body silvering, growth, metabolism, and behavior changes (Dickhoff et al. 1982b). Factors which have been shown to influence concentrations in salmonids during smoltification include phase of lunar cycle (Grau et al. 1981; Yamauchi et al. 1984; McCreery 1987), body size, temperature and photoperiod (Pereira and Adelman 1985), rearing density (Patiño et al. 1986), water flow rates (Youngson and Simpson 1984; Youngson et al. 1985) and chemical changes in the water (Dickhoff et al. 1982a; Specker and Schreck 1984; Grau et al. 1985; Lin et al. 1985; Nishioka et al. 1985).

Two components of streams which change during spring runoff are water chemistry and velocity. The stimulation of plasma thyroxine surges by changes in water chemistry and/or increased flow rate may be an adaptation which allows juvenile salmon to undergo synchronous smoltification, rapidly prepare for life in salt water, and use increased stream velocity of spring runoff to speed their downstream migration. In most hatcheries, however, conditions of water chemistry, temperature, and flow rate are constant. Nishioka et al. (1985) suggested that such uniform conditions may provide insufficient stimulation for eliciting changes in thyroid function and thus have negative consequences on the smoltification process.

Previous experiments which have shown an effect of novel water on plasma thyroxine levels have involved transporting fish and movement from one tank to another (Dickhoff et al. 1982b; Grau et al. 1985; Lin et al. 1985) or by addition of chemicals (Specker and Schreck 1984; Nishioka et al. 1985). In this study the separate influences of changes in water chemistry and flow rate were assessed in chinook salmon subjected to either artificial or natural photocycles in a single hatchery. The changes in water chemistry were accomplished by rearing salmon initially on well water and later mixing well with lake water of a distinct chemical nature.

Methods

Chinook salmon sampled in this study were raised at Blue Dog Lake State Fish Hatchery, Waubay, South Dakota from eggs taken from adults returning to the Little Manistee River, Michigan of stock originating from the Toutle River, Washington. Six groups of salmon were raised in 8.9°C well water at 114 litres per minute (L/min) in indoor raceways with full artificial illumination provided from 08:00–16:30 and one-half intensity illumination for the remainder of the day/night cycle (8.5L:15.5D photocycle). On 25 March, 1988, three of these groups were moved to outdoor raceways with a naturally increasing photocycle but with water characteristics and fish density identical to the indoor raceways. The natural photoperiod on this date was 12.5L:11.5D. The indoor control group was maintained under the initial conditions of water source, flow rate, and photoperiod throughout the experiment. The outdoor control was also held under the initial water source and flow conditions but with a naturally increasing photocycle. On 30 March the water sources or flow rates were changed on the four experimental groups. The indoor and outdoor increased flow groups continued to receive well water but at 230 L/min. The indoor and outdoor novel water groups received a 1:1 mixture of well and Blue Dog Lake water at 114 L/min. Fish from the indoor control were transported to the Whitlocks Bay Spawning and Imprinting Station, Lake Oahe, South Dakota, on 23 April where they were held for 2 wk, prior to release, in completely novel water.

Blood samples were collected from 30 fish per treatment group (180 fish) on 18, 26, and 31 March and 2, 4, 7, 9, 12, 14, 16, and 22 April. In addition, fish transferred to the Whitlocks Bay Station (indoor control group) were also sampled on 30 April and 8 May, 7 and 15 days after transfer. Samples were collected from fish taken from the middle of the raceway and placed in a 19 L bucket. Individual fish were randomly taken from the bucket and stunned by a blow to the head. Blood was collected in heparinized capillary tubes after severing the tail at the caudal peduncle. No more than 10 fish were removed from each bucket and sampling of all 10 fish took 8 min or less. Blood samples were then centrifuged and the plasma separated and stored at −20°C until analysis.

Plasma thyroxine concentration was assayed using solid phase radioimmunoassay kits (Micromedic Systems, Horsham, PA). The effectiveness of the use of these kits to determine hormone levels in fish has been previously reported (Spieler and Noeske 1981; Noeske and Spieler 1983). Cross-reactivity with 3,3′,5-triiodothyronine is 1.3% in these assay kits. Intra-assay variation was estimated to be 9.96% as determined by calculation of the coefficient of variation of 20 identical duplicate samples of known thyroxine levels from the midpoint of the standard curve. Nonspecific binding was 0.3% of the total counts added. All samples were analyzed in a single assay to eliminate the problem of interassay variability. Standards were prepared by adding known quantities of plasma thyroxine to chinook salmon plasma previously charcoal stripped of all endogenous thyroid hormones. Duplicate 5 μL undiluted samples and standards were assayed and the bound fraction counted with a Beckman 5500 Gamma Counter. Estimates of sample plasma thyroxine content were determined from a linear regression between appropriate adjacent points on the standard curve. Due to the limited plasma volume from each fish sampled and the predominance of plasma thyroxine analysis in previous studies, only plasma thyroxine was assayed in this study.

Mean plasma thyroxine level for each experimental group was compared with the appropriate control group. SAS for Personal Computers (SAS Institute Inc., Cary, NC) was used to conduct ANOVA and multiple comparison procedures. Means were compared using the Student–Newman–Keuls multiple range test at $\alpha = 0.05$.

Results

Changes in plasma thyroxine levels in these salmon were primarily associated with the experimental treatment to which they were exposed. Patterns of change in plasma thyroxine levels were similar in the two photoperiods but changes in hormone levels in the natural photoperiod groups were greater in magnitude. The two novel water groups showed a significant increase in plasma thyroxine levels over the period of the experiment and experienced the greatest increase in plasma thyroxine levels. The increased flow groups had the least variable and generally the lowest plasma thyroxine levels but were overall not significantly different from the controls.

Effects of Novel Water

The introduction of a novel water source caused a significant increase in plasma thyroxine levels (Fig. 1). From 18 March through 7 April, no significant differences in mean plasma thyroxine levels were detected between a novel water group and its control nor was any difference observed in mean plasma thyroxine levels within either the experimental or control groups. However, on 9 April, 10 d after the introduction of novel water, plasma thyroxine levels in both the indoor and outdoor novel water groups increased to a level greater than any other sample during the experiment (17.7 and 25.5 ng/mL, respectively). Plasma thyroxine levels in the indoor novel water group were significantly greater than those of the control on 9 and 22 April. In the outdoor novel water group plasma thyroxine levels were significantly greater than those of the control on 9, 14, and 22 April.

Plasma thyroxine levels were also measured in salmon from the indoor control after transfer to the Whitlocks Bay Station. Plasma thyroxine levels were 24.7 and 15.0 ng/mL 7 and 15 d after transfer, respectively. Both of these levels were significantly higher than any recorded for this group at Blue Dog Hatchery.

Effects of water flow

Doubling the rate of water flow in the raceways failed to affect plasma thyroxine levels in these chinook salmon as nei-

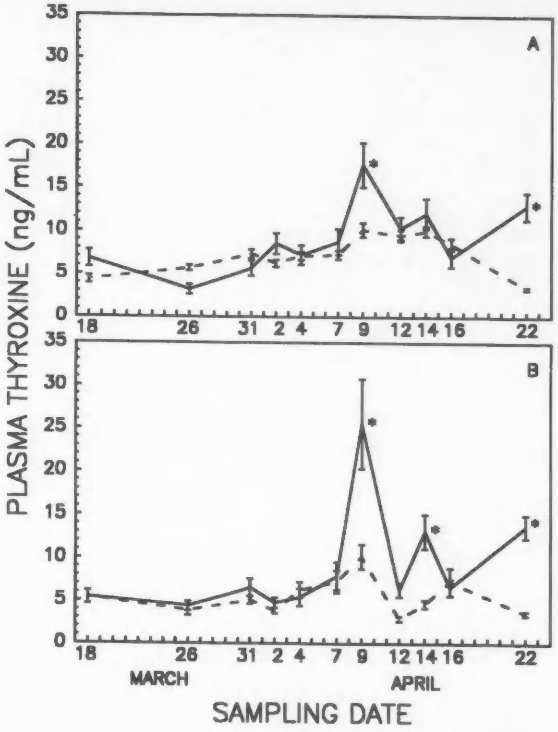

FIG. 1. Plasma thyroxine levels of novel water (solid line) and control (dashed line) groups held indoor (A) and outdoor (B) at Blue Dog Lake State Fish Hatchery in 1988. Asterisk (*) indicates a significant difference in plasma thyroxine levels for that date (α = 0.05).

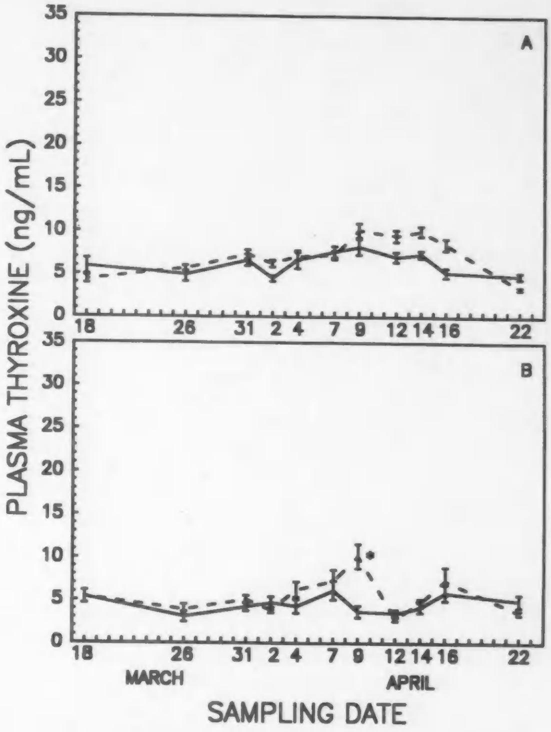

FIG. 2. Plasma thyroxine levels of increased flow (solid line) and control (dashed line) groups held indoor (A) and outdoor (B) at Blue Dog Lake State Fish Hatchery in 1988. Asterisk (*) indicates a significant difference in plasma thyroxine levels for that date (α = 0.05).

ther increased flow group showed a significant peak in plasma thyroxine level over the sampling period (Fig. 2). The plasma thyroxine levels of both the indoor and outdoor increased flow groups were consistently lower than those of the respective control. However, only on 9 April were levels in the outdoor increased flow group signficantly lower than those of the outdoor control.

Discussion

Our results clearly indicate the stimulatory influence of changes in water chemistry on plasma thyroxine levels in chinook salmon undergoing smoltification. Exposure of fish to a mixture of water sources resulted in significantly elevated levels of plasma thyroxine. In contrast, increasing flow rate of a uniform water chemistry did not influence plasma thyroxine levels during smoltification. When the fish were maintained indoor at Blue Dog Hatchery with a uniform water supply there were minimal significant changes in plasma thyroxine levels during the smoltification period. These results agree with previous results on the effects of novel water chemistry on smoltification (Specker and Schreck 1984; Grau et al. 1985; Lin et al. 1985; Nishioka et al. 1985; Lin et al. 1988) but conflict with those indicating a stimulatory effect of increased water flow on plasma thyroxine levels (Youngson and Simpson 1984; Youngson et al. 1985).

Previous results on the influence of a novel water source have been confounded by the transfer of fish, possibly indicating a disturbance effect. In 1987 we sampled chinook salmon weekly from 3 March–4 May at Blue Dog Hatchery. Plasma thyroxine levels did not significantly rise during this period. However, in both 1987 and 1988 plasma thyroxine increased significantly after salmon were transferred from Blue Dog Hatchery to the Whitlocks Bay Spawning and Imprinting Station (T. L. Hoffnagle and A. J. Fivizzani, unpubl. data). Grau et al. (1985) also reported rapid increases in plasma thyroxine levels when coho salmon were transferred from one freshwater tank to another; this elevation of hormone level was maintained over the 48-h duration of the experiment. Lin et al. (1985) transported coho salmon from a hatchery to the laboratory and then into different types of tanks. These investigators found increases in plasma thyroxine only when the fish were transferred into a different type of tank and only before the new moon-associated plasma thyroxine peak in this stock. In the present hatchery experiment, which excluded transfer of fish, 10 d of exposure to the lake–well water mixture were required to cause an elevation in plasma thyroxine comparable to that observed by Lin et al. (1985) after 24 h. Dickhoff et al. (1982a) transported fish to a new location and into either novel water or water from their original location. Only fish which were exposed to a novel water source showed an increase in plasma thyroxine concentration. This demonstrated that it is unlikely that transport stress is a cause of the increases in plasma thyroxine levels seen by Lin et al. (1985) and in our transfers of fish to the Whitlocks Bay Station. Specker and Schreck (1980) also reported that transportation of coho salmon did not affect thyroxine levels.

Some previous studies have investigated the nature of chemical changes required to stimulate a rise in plasma thyroxine levels. Nishioka et al. (1985) changed water chemistry by adding three salt treatments over the period of 1 mo which produced a plasma thyroxine surge in yearling coho salmon. However, when seawater and amino acids were added to the water, plasma thyroxine levels in the fish were unaffected except on new moon dates. Specker and Schreck (1984) also used salts to change water chemistry. They found elevated plasma thyroxine levels in coho salmon 1 wk after exposure to 12–24 mM NaCl but exposure of fish to 6–12 mM $CaCl_2$ had no effect during their 3 wk experiment. Chinook salmon transferred to surface net pens in Lake Sakakawea, North Dakota developed plasma thyroxine levels five-fold greater than those of fish maintained in a hatchery receiving hypolimnion water from the same reservoir (McCreery 1987).

Our results indicate that smoltification of chinook salmon is not stimulated by increased flow. Yamauchi et al. (1985) saw an increase in downstream migratory behavior of masu (cherry) salmon (*Oncorhynchus masou*) 3 d after a heavy rainfall had exposed the fish to novel water, but not increased flow, in an artificial stream. Youngson and Simpson (1984), however, reported a positive correlation between serum plasma thyroxine levels and stream discharge rate in smolting Atlantic salmon (*Salmo salar*). This correlation does not demonstrate a cause and effect relationship since changes in water chemistry during the period of increased flow were not monitored in their study. However, Youngson et al. (1985) showed that plasma thyroxine levels in Atlantic salmon increased 6 h after exposure to increased flow rates.

Our results support the hypothesis of Nishioka et al. (1985) that hatchery rearing of young salmonids may not provide a sufficiently changing environment for stimulation of the thyroid gland and thus induction of smoltification. Most previous studies were conducted at hatcheries where salmon were raised outdoors, exposing them to natural photoperiods and lunar cycles. Blue Dog Hatchery was designed as a warm and cool water hatchery where fry, particularly of northern pike (*Esox lucius*) and walleye (*Stizostedion vitreum vitreum*) are held indoor for only a short period beyond hatching. At Blue Dog Hatchery the artificial photoperiods are short and unchanging and uniform water flow and chemical conditions are maintained. The lack of environmental changes there may be the basis of the lack of smoltification-associated increases in plasma thyroxine in non-experimental (control) chinook salmon. Our observations of increasing plasma thyroxine levels after transfer of salmon to the Whitlocks Station may result from previously understimulated fish being exposed to environmental cues which trigger smoltification-associated thyroid stimulation.

Transfer to novel water may mimic the repeated stimuli of novel water chemistry from incoming tributaries encountered as fish move downstream and may be a mechanism for imprinting (Grau et al. 1985). Scholz (1982) injected coho salmon with thyroid stimulating hormone (TSH) and exposed them to a synthetic chemical upon which to imprint. Fish treated with TSH had higher return rates than fish receiving injections of saline or ACTH, presumably due to elevated levels of plasma thyroxine in the TSH-injected fish. Similarly, we anticipate a higher return rate to the Whitlocks Bay Station of adults of groups which attain greater plasma thyroxine levels before release.

The return of sexually mature chinook salmon to the Whitlock Bay Station during its first 5 yr of operation has been lower than anticipated. This low return may be due to the absence of appropriate environmental stimuli prior to the time of normal smoltification in these fish. The results of this study, as well as others, underscore the importance of water chemistry changes, and perhaps of other cues, in the induction of normal smoltification. Long-term experiments on adult return rates are in progress and future experiments will be performed to test this hypothesis.

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References

DICKHOFF, W. W., D. S. DARLING, AND A. GORBMAN. 1982a. Thyroid function during smoltification of salmonid fish. Gunma Symposia on Endocrinology 19: 45–61.

DICKHOFF, W. W., C. SULLIVAN, AND C. V. W. MAHNKEN. 1982b. Methods of measuring and controlling the parr to smolt transformation (smoltification) of juvenile salmon. *In* C. J. Sinderman [ed.] Proceedings of the 11th U.S.–Japan meeting on aquaculture, salmon enhancement. Tokyo, Japan.

GRAU, E. G., W. W. DICKHOFF, R. S. NISHIOKA, H. A. BERN, AND L. C. FOLMAR. 1981. Lunar phasing of the thyroxine surge preparatory to seaward migration of salmonid fish. Science (Wash., DC.) 211: 607–609.

GRAU, E. G., A. W. FAST, R. S. NISHIOKA, AND H. A. BERN. 1985. The effects of transfer to novel conditions on blood thyroxine in coho salmon. Aqaculture 45: 377.

LIN, R. J., R. J. RIVAS, E. G. GRAU, R. S. NISHIOKA, AND H. A. BERN. 1985. Changes in plasma thyroxine following transfer of young coho salmon (*Oncorhynchus kisutch*) from fresh water to fresh water. Aquaculture 45: 381–382.

LIN, R. J., T. F. CROSS, C. P. R. MILLS, R. S. NISHIOKA, E. G. GRAU, AND H. A. BERN. 1988. Changes in plasma thyroxine levels during smoltification in hatchery-reared one-year and two-year Atlantic salmon (*Salmo salar*). Aquaculture 74: 369–378.

MCCREERY, T. P. 1987. Hormones and behavior in chinook salmon (*Oncorhynchus tshawytscha*) and steelhead trout (*Salmo gairdneri*) during smoltification. Masters thesis, University of North Dakota, Grand Forks, ND.

NISHIOKA, R. S., G. YOUNG, H. A. BERN, W. JOCHIMSEN, AND C. HISER. 1985. Attempts to intensify the thyroxine surge in coho and king salmon by chemical stimulation. Aquaculture 45: 215–225.

NOESKE, T. A., AND R. E. SPIELER. 1983. Photoperiod and diel variations of serum cortisol, thyroxine and protein in goldfish, *Carassius auratus* L. J. Fish Biol. 23: 705–710.

PATIÑO, R., C. B. SCHRECK, J. L. BANKS, AND W. S. ZAUGG. 1986. Effects of rearing conditions on the developmental physiology of smolting coho salmon. Trans. Am. Fish. Soc. 115: 828–837.

PEREIRA, D. L., AND I. R. ADELMAN. 1985. Interactions of temperature, size and photoperiod on growth and smoltification of chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture 46: 185–192.

SCHOLZ, A. T. 1982. Hormonal regulation of smolt transformation and olfactory imprinting in coho salmon. *In* E. L. Brannon and E. O. Salo. [ed.] Salmon and trout migratory behavior symposium. School of Fisheries, University of Washington, Seattle, WA.

SPECKER, J. L., AND C. B. SCHRECK. 1980. Stress responses to transportation and fitness for marine survival in coho salmon (*Oncorhynchus kisutch*) smolts. Can. J. Fish. Aquat. Sci. 37: 765–769.

SPECKER, J. L., AND C. B. SCHRECK. 1984. Influence of ambient salts on plasma thyroxine concentrations in juvenile coho salmon. Trans. Am. Fish. Soc. 113: 90–94.

SPIELER, R. E., AND T. A. NOESKE. 1981. Timing of a single meal and diel variations of serum thyroxine, triiodothyronine and cortisol in goldfish, *Carassius auratus*. Life Sci. 28: 2939–2944.

YAMAUCHI, K., N. KOIDE, S. ADACHI, AND Y. NAGAHAMA. 1984. Changes in seawater adaptability and blood thyroxine concentrations during smoltification of the masu salmon, *Oncorhynchus masou*, and the amago salmon, *Oncorhynchus rhodurus*. Aquaculture 42: 247–256.

YAMAUCHI, K., M. BAN, N. KASAHARA, T. IZUMI, H. KOJIMA, AND T. HARAKO. 1985. Physiological and behavioral changes occurring during smoltification in the masu salmon, *Oncorhynchus masou*. Aquaculture 45: 227–235.

YOUNGSON, A. F., AND T. H. SIMPSON. 1984. Changes in serum thyroxine levels during smolting in captive and wild Atlantic salmon, *Salmon salar* L. J. Fish. Biol. 24: 29–39.

YOUNGSON, A. F., D. C. B. SCOTT, R. JOHNSTONE, AND D. PRETSWELL. 1985. The thyroid system's role in the downstream migration of Atlantic salmon (*Salmon salar*) smolts. Aquaculture 45: 392–393.

Effect of Dietary Ascorbate on the Concentration of Tissue Ascorbic Acid, Dehydroascorbic Acid, Ascorbic Sulfate, and Activity of Ascorbic Sulfate Sulfohydrolase in Rainbow Trout (*Oncorhynchus mykiss*)

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Dabrowski, K., R. Lackner, and C. Doblander. 1990. Effect of dietary ascorbate on the concentration of tissue ascorbic acid, dehydroascorbic acid, ascorbic sulfate, and activity of ascorbic sulfate sulfohydrolase in rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 47: 1518–1525.

The concentrations of ascorbic acid in several tissues of rainbow trout (*Oncorhynchus mykiss*) are significantly influenced by various dietary treatments. Ascorbic acid was taken up readily by erythrocytes, kidney, liver, intestine, spleen, and brain in fish fed an ascorbate supplemented diet (AA group), the concentration being from 1.5 to 14.8-fold higher than in fish fed a diet lacking ascorbate (control group). In fish fed a diet supplemented with an equimolar amount of ascorbic acid in the form of ascorbic sulfate (AS group) the ascorbic acid concentrations in kidney, intestine, and erythrocytes were significantly elevated above those of the control group. Ascorbic sulfate was found in kidney, liver, and intestine of the AS group, but not in other groups. In fish fed a diet devoid of vitamin C the ascorbic acid concentrations in kidney, liver, intestine, and spleen were signficantly lower than in fasting fish over the same period of time (28 d), suggesting a high demand for vitamin C in an actively feeding animal. Salmonid fish are therefore probably unable to utilize ascorbic sulfate sufficiently to prevent the appearance of vitamin C deficiency, and thus resemble scurvy-prone mammals in this respect.

Dans plusieurs tissus de la truite arc-en-ciel (*Oncorhynchus mykiss*), les concentrations d'acide ascorbique varient nettement selon le régime alimentaire. L'acide ascorbique a été promptement absorbé par les érythrocytes, les reins, le foie, l'intestin, la rate et le cerveau des poissons nourris d'aliments enrichis d'ascorbate (groupe AA); ainsi, sa concentration était de 1,5 à 14,8 fois plus élevée que chez les poissons nourris d'aliments libres d'ascorbate (groupe témoin). Chez les poissons nourris d'aliments enrichis d'une quantité équimolaire d'acide ascorbique sous la forme de sulfate d'acide ascorbique (groupe AS), les concentrations d'acide ascorbique dans les reins, l'intestin et les érythrocytes étaient nettement plus élevées que chez le groupe témoin. Le sulfate d'acide ascorbique était présent dans les reins, le foie et l'intestin du groupe AS mais non chez les autres groupes. Chez les poissons nourris d'aliments libres de vitamine C, les concentrations d'acide ascorbique dans les reins, le foie, l'intestin et la rate étaient nettement inférieures à celles relevées chez les poissons à jeun au cours de la même période (28 jours), ce qui porte à croire à un besoin donc probable que l'assimilation du sulfate d'acide ascorbique n'est pas suffisante chez les salmonidés pour empêcher une carence en vitamine C. Dans ce sens, ils ressemblent aux mammifères susceptibles au scorbut.

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The requirement for dietary ascorbic acid (vitamin C) is not a common characteristic of vertebrates (Chatterje 1973); whereas it is required by salmonid fish (Halver 1985), some species of cyprinids are claimed to be able to synthesize ascorbic acid in their liver and/or kidney (Yamamoto et al. 1978; Thomas et al. 1985). Investigations into the role of ascorbic acid in vertebrate metabolism have focused most recently on the striking difference between fish and scurvy-prone mammals in respect to absorption, excretion, transport into tissues, and utilization of ascorbic sulfate (Tucker and Halver 1984, 1986). Some reports claimed that in the guinea pig or monkey, ascorbic sulfate (AS) given either orally or parenterally had no antiscorbutic activity (Kuenzig et al. 1974; Machlin et al. 1976), whereas Mumma et al. (1972) demonstrated that the guinea pig fed a diet supplemented with AS gained weight, and showed no pathological manifestations. It has been suggested that fish, salmonids in particular, differ from mammals with respect to vitamin C metabolism in being able to synthesize ascorbate-2-sulfate and to use this compound as a storage form (Tucker and Halver 1986). Hepatic ascorbic sulfate sulfohydrolase was isolated and biochemically characterized in salmonid and other fish (Benitez and Halver 1982; Daniel et al. 1987). Reports concerning utilization of dietary AS by fish are, however, contradictory. It was demonstrated in two fish species that prolonged feeding with AS led to a considerably greater depletion in tissue ascorbic acid than a diet supplemented with the equimolar amount of ascorbic acid (AA) (Murai et al. 1978; Soliman et al. 1986). It was also reported that salmonid fish need at least 160 mg·kg^{-1} of AS to prevent scurvy whereas only 40 mg·kg^{-1} of AA is recommended (Halver et al. 1975).

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TABLE 1. Formulation of basal diet fed to rainbow trout.

Ingredient[a]	Concentration[b] (g·kg^{-1} dry diet)
Casein	400
Gelatin	100
Dextrin	254.6
Carboxymethyl cellulose (CMC)	20
Soya oil[c]	60
Cod-liver oil[c]	60
L-methionine	2
L-tryptophan	0.4
Mineral mixture[d]	80
Vitamin mixture[e]	10
NaCl	5
Choline chloride	2
Chromic oxide	5

[a] Dietary ingredients were commercially obtained. The vitamin-free casein, gelatin, dextrin, CMC, BHT and free amino acids were from Sigma Chemical Co., St. Louis Missouri, USA. Chromic oxide was from Merck, Darmstadt, F.R.G.

[b] The basal diet adds up to 999 g. The space is left for AA, AS or dextrin addition.

[c] Cod-liver oil and soya oil were from local commercial suppliers.

[d] The mineral supplement provided the following (g·kg^{-1} of dry diet): $CaHPO_4 \cdot 2H_2O$ 18, $CaCO_3$ 19, KH_2PO_4 14, $NaHCO_3$ 8.88, $MnSO_4 \cdot H_2O$ 0.35, $FeSO_4 \cdot 7H_2O$ 0.5, $MgSO_4$, 3, KIO_3 0.01, $CuSO_4 \cdot 5H_2O$ 0.03, $ZnCO_3$ 0.15, $CoCl_2 \cdot 6H_2O$ 0.002, $Na_2MoO_4 \cdot 2H_2O$ 0.008, Na_3SeO_3 0.002.

[e] The vitamin supplement provided the following amounts per kg of dry diet: tocopheryl acetate 150 mg, biotin 5 mg, mesoinositol 400 mg, nicotinic acid 10 mg, thiamin HCl 30 mg, Ca-pantothenate 100 mg, folic acid 10 mg, menadione 10 mg, pyridoxine-HCl 15 mg, vitamin B_{12} 0.04 mg, vitamin D_3 20 mg (500 000 I.U. in 1 g), vitamin A (500 000 I.U. in 1 g) 30 mg, riboflavin 25 mg, BHT 50 mg. The vitamins were from Hoffmann La Roche, Basel, Switzerland.

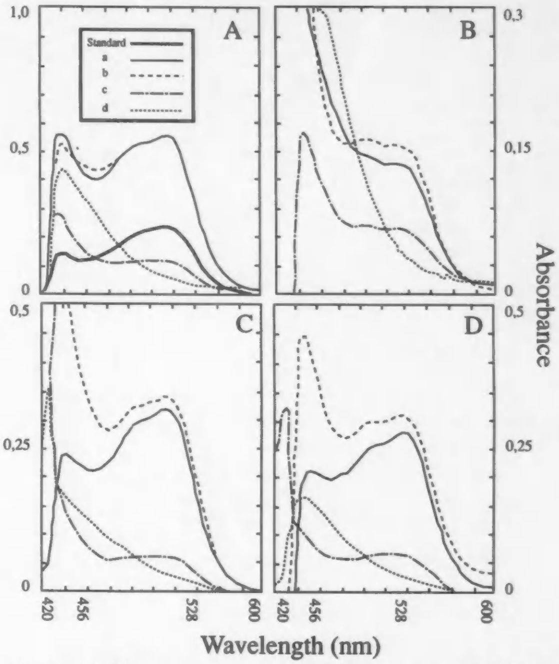

FIG. 1. Absorbance spectra of DNPH–derivatives formed during ascorbate analysis in tissue extracts. Solid thick line, standard (10 μg·mL^{-1}); a, total ascorbate; b, total ascorbate plus ascorbate liberated from AS after KBr0$_3$ hydrolysis; c, dehydroascorbate; d, ascorbate destroyed by preincubation at 90°C. A and B, intestinal extracts from fish fed on the AA- and AS-supplemented diets, respectively. C and D, brain extracts from fish fed on the AA- and AS-supplemented diets.

We have therefore investigated several ways in which salmonid fish might utilize AS as a source of AA (Dabrowski and Kock 1989). The objectives of this study were to identify the major forms of AA in salmonid fish tissues, explain the possible role of glutathione in maintaining reduced/oxidized ascorbate ratio, and measure storage and distribution of AA, dehydroascorbate and AS in fish maintained on various feeding regimes.

Materials and Methods

Experiment

The feeding experiment was conducted with rainbow trout (*Oncorhynchus mykiss*) yearlings. They were obtained from a Thauer Fish Hatchery, Tirol County, Austria, and kept for 2 wk in the laboratory prior to the experiment.

They were fed a rainbow trout pelleted diet (Almå Futter, Kempten, F.R.G.), which contained 250 μg·g^{-1} of total AA (measured in our laboratory). At the start of the trial, fish were divided into groups of 10 individuals, and kept in tanks with several compartments, supplied with aerated city water, 10° ± 1.0°C. Each compartment had a volume of 250 L; dissolved oxygen was maintained close to saturation.

The diets were prepared from a formula similar to that used by Hughes et al. (1981) (Table 1). Ascorbic acid (M.W. 176.1) and AS (M.W. 381.8) were from F. Hoffmann–La Roche, Basel Switzerland; dehydroascorbic acid was from Aldrich–Chemie, Steinheim, F.R.G. Wet pellets, processed on a laboratory mill, were immediately frozen (−80°C) and freeze-dried within the next 24–48 h. The diets were stored in sealed bags in a freezer (−18°C) until required for feeding. Diets were supplemented with either 0 (diet 1), 500 mg AA·kg^{-1} (diet 2), or 1084 mg AS (dipotassium ascorbic-2-sulfate-dehydrate)·kg^{-1} (equivalent to 500 mg AA·kg^{-1}) (diet 3). One group was fasted for the same period of 28 d.

Five fish were anesthetized before the trial and after its termination; from each of the experimental groups blood was collected from the caudal vessel. The fish were then killed, and specific organs (liver, kidney, pyloric caeca region of intestine, spleen, and brain) were removed and quick-frozen (−80°C). Blood was centrifuged (11 000 *g*, 3 min, 4°C) and the plasma and erythrocyte fractions were separated and frozen.

AA and AS Determination

Frozen tissue was homogenized in cold extraction solution (5% trichloroacetic acid in 250 mm HC10$_4$ containing 0.08% EDTA). AA and AS were analyzed according to the methods already described (Carr et al. 1983; Terada et al. 1978) with the following modifications: the background level determination of the interfering substances which form the color complexes with dinitrophenyl-hydrazine (DNPH) was subtracted from the level of total and dehydroascorbic acid (DA). Either directly or after hydrolysis of AS with 1% $KBrO_3$ (Terada et al. 1978) parts of the extract were kept for 1.5 h in a water bath at 90°C. This procedure destroys the AA. To exclude all possible interference in all types of tissue the absorbance from 600 to 420 nm was recorded with a Beckman DU-6 spectrophotometer and the difference in absorbance at 524 nm was measured directly on the scans (Fig. 1) (Dabrowski and Hinterleitner 1989).

Enzyme Assays

Arylsulfatase (AR-ase)

Tissue samples (liver and pyloric caeca) were homogenized in Tris-HCI buffer, 50 mM pH 7.5. The homogenate was centrifuged for 20 min at 15 000 *g* at 4°C and the supernatant dialyzed overnight against Tris-buffer.

The procedure for determining AR-ase activity was similar to that previously described (Carlson et al. 1977) but modified for kinetic studies. Twenty μL of the enzyme were added to 480 μL of a solution of 3 mM *p*-nitrocatechol sulfate (NCS) in 0.1 M 2/N-morpholino-ethanesulfonic acid (MES) buffer at pH 6.2 and incubated at 25°C.

Every 5 min for 20 min 50 μL of the mixture were placed into 450 μL of 0.2 M NaOH. The increase in absorbance at 510 nm was measured for 20 min. P-nitrocatechol was used to establish the standard curve for the assay and an extinction coefficient of $n = 25\ \mathrm{cm^2/mM}$ was used.

Ascorbic sulfate sulfohydrolase (ASS-ase)

Ascorbic acid liberated by enzymatic hydrolysis was measured in the solution containing 600 μL of 0.1 M sodium acetate buffer, pH 5.5, 200 μL of 100 mM AS, 100 μL of 0.2% albumin in sodium acetate buffer, and 100 μL of crude, dialyzed enzyme. Appropriate blanks with no enzyme were run simultaneously and the reaction was stopped by adding 200 μL of the reaction mixture to 800 μL of TCA–PCA–EDTA solution (see AA determination in Dabrowski and Hinterleitner 1989) at time 0 and after 8 h. For kinetic studies, the volume of the reaction mixture was increased and triplicate subsamples were removed at various times between 0 and 8 h (data not shown). Under conditions used in the present studies, the reaction remained linear.

Dehydroascorbate reductase (DA-ase)

Tissue samples (liver, kidney, and pyloric caeca) were homogenized in 0.65% NaCl solution in a glass homogenizer. The procedure was similar to that previously described (Stahl et al. 1983) and was based on the change of absorbance at 265 nm associated with the generation of AA. The standard reaction mixture contained 800 μL of 50 mM phosphate buffer pH 6.7, 50 μL of 25 mM dehydroascorbic acid (DA), 50 μL of reduced glutathione (GSH), 50 μL 2.5 mM EDTA, and 50 μL of enzyme. The reaction was initiated by adding enzyme to the solution followed immediately by the addition of DA.

A blank was run simultaneously without enzyme (chemical reduction). The increase in absorbance at 265 nM was recorded at 25°C for 4–5 min. Instead of DA, 50 μL of 10–50 $\mu\mathrm{m{\cdot}mL^{-1}}$ AA were placed in a cuvette with the reaction mixture and a standard curve was established for these assay conditions.

Glutathione (GSH) concentration in the extract prepared for AA determination (Dabrowski and Hinterleitner 1989) was assayed by the colorimetric procedure (Nimmo 1985). This method measured the acid soluble thiols, but an enzymatic test proved that GSH constituted up to 90% of these compounds in fish tissue.

Protein was analysed (Lowry et al. 1951) after precipitation of the enzyme extract with 5% TCA and resuspension of the sediment in 1 M NaOH.

Metal Analysis

Metals in plasma were determined after predigestion in a nitric–perchloric acid mixture by flame atomic spectrophotometry with a Perkin Elmer 2380 apparatus.

Statistical Analysis

Data are expressed as mean values plus or minus standard deviation (SD). Statistical significance of differences between means was determined using Student's *t*-test and Duncan's multiple range test ($P<0.05$).

Results

During the 28 d trial the fish not receiving food lost 11.3% body weight, whereas those receiving diets 1 (no AA), 2 (with AA), and 3 (with AS) gained 14.1, 26.3, and 17.6% body weight, respectively (Table 2). The liver-somatic index was significantly higher ($P<0.01$) in fed than in starved fish, although the type of diet received made no significant difference.

In the case of intestinal tissue from the group fed a diet supplemented with AA there was no difference between the total ascorbate and the sum of total ascorbate plus AS (Fig. 1A). Determination of DA alone (DCIP oxidation step omitted) yielded a relatively low absorbance peak at 524 nm suggesting that most of the ascorbate in this tissue was in the reduced form. The procedure, which involved ascorbate degradation, suggested that nearly 50% of absorbance at 524 nm came from the interfering substances which formed a complex with DNPH showing maximum absorbance at 440 nm. An increase in the amount of ascorbic acid liberated was found in the extract from intestine of fish fed diet 3 (Fig. 1B) after it was preincubated with $KBr0_3$. The latter substance hydrolyses AS at room temperature. The results suggested that, in fish fed an AS-supplemented diet, AS accumulated in the intestine. On the basis of the data presented in Fig. 1C and D the assumed presence of AS in trout brain (and other tissues) was dismissed. Hydrolysis with $KBr0_3$ frequently led to increased absorbance at 524 nm due to interfering substances.

In trout, before the commencement of the trial, the highest level of ascorbate was found in the brain, followed by kidney and liver with almost half this amount (Table 3). The major part of ascorbate in the kidney was in the oxidized form, whereas in brain and liver the reduced ascorbate dominated.

The concentration of ascorbate in the plasma of fish 16 h after the last feeding reflected the dietary supplementation of AA and AS (Table 4). The plasma Cu level was significantly higher ($P < 0.05$) in the fish group fed diet 2 and 3, whereas the zinc concentrations in plasma were only slightly influenced by the dietary treatment. The addition of AA to the diet significantly changed the ascorbate distribution in trout tissue (Table 5). Ascorbate entered all the tissues and the increase in comparison to the pre-experimental level (Table 3) was several-fold in all tissues excluding brain. Kidney maintained the low level of reduced ascorbate in the total pool in the group fed diet 2, but the proportion of the oxidized DA was still higher in the rest of the treatments. This tendency for a much higher percentage of the reduced form in fish fed AA-supplemented diet

TABLE 2. Ascorbic acid (AA) or ascorbic sulfate (AS) intake of rainbow trout and initial and final individual body weights, liver-somatic index, and feed: weight gain ratios.

	Treatment			
	Fasting	Diet 1	Diet 2 (AA)	Diet 3 (AS)
AA or AS intake $mg \cdot kg^{-1}$ wet $wt \cdot d^{-1}$ on ascorbate molar basis	0.0	0.0	2.85	3.05
Initial, ^{1},^{2}g	67.8 ± 10.8^{a}	62.9 ± 12.4^{a}	71.1 ± 13.0^{a}	63.8 ± 10.1^{a}
Final^{1},g	60.1 ± 8.2^{a}	71.8 ± 17.7^{a}	89.8 ± 21.8^{a}	75.0 ± 19.8^{a}
Final liver-somatic Index (% body wt)	1.08 ± 0.24^{a}	1.55 ± 0.21^{b}	1.82 ± 0.28^{b}	1.8 ± 0.26^{b}
Feed:wt gain ratio	0.0	1.79	0.91	1.42

[1] Mean ± SD, for 10 fish per treatment.

[2] Groups with common superscript letters within horizontal line are not significantly different ($P<0.05$) on the basis of Duncan test.

TABLE 3. Total ascorbic acid (AA) and dehydroascorbic acid (A) concentrations in tissues of rainbow trout.

Tissue	AA ($\mu g \cdot g^{-1}$ or	DA $\mu g \cdot mL^{-1}$)	(%)^{a}
Plasma^{b}	3.18 ± 2.2	0.7 ± 0.4	(78.0)
Erythrocytes	6.2 ± 0.6	6.3 ± 0.3	(0.0)
Kidney	83.4 ± 32.3	53.3 ± 27.4	(36.1)
Liver	72.7 ± 8.4	21.9 ± 8.7	(70.0)
Brain	138.4 ± 15.8	35.3 ± 9.4	(74.5)

[a] Percentage of the reduced form.

[b] Mean ± SD; $n=5$.

was consistent in all tissues analysed. Ascorbate was significantly higher ($P < 0.01$) in kidney and pyloric caeca intestinal region of trout fed an AS-supplemented diet than in fish offered a diet lacking ascorbate. However, in fasted fish and those fed diet 1 or 3, ascorbate levels in liver and kidney decreased below the levels found at the beginning of the experiment (Table 3).

In fish fed diet 1 (no AA), in comparison to the fasting group, the decrease in ascorbate level in all tissues (excluding brain) was signficant ($P < 0.01$). The turnover time of ascorbate, or in other words, the metabolic requirement of AA, is considerably higher in actively feeding fish than in fasting fish; the total difference in body weight (25.4%) (Table 2) can only partly account for the differences in tissue ascorbate where actual ascorbate concentration decreased by 60% in liver, kidney, and intestine (Table 5).

There is much higher consistency of GSH concentration due to type of tissue than to dietary treatment (Table 6). Furthermore, GSH concentration in a given tissue and group on various dietary treatments does not correspond to the proportion of DA in the total ascorbate pool (Table 5). GSH concentration in brain and kidney increased during the trial (Table 6). Despite a high level of GSH in erythrocytes compared to plasma, the ascorbate in the former tissue was exclusively in the oxidized form (Table 5). The GSH concentrations in plasma were only slightly influenced by the dietary treatment.

There was no reduction of DA by trout tissue extract without added GSH in the assay. Under the conditions used in the present studies, chemically formed AA within the first 4 min of the reaction amounted to 26.6 μM in comparison to 15.4 μM produced by the added enzyme. No activity of DA-ase was found in the intestine (Table 7). Despite a greater variation among the individual fish within the experimental group, there was a tendency towards increasing activity of DA-ase in fish liver and kidney with increased AA supply. Enzyme activity corresponded, in this respect, to the partitioning of the reduced form of ascorbate in the total pool (Table 5). AR-ase was significantly higher ($P < 0.01$) in the liver of fasting trout than in fed groups (Table 7). In fish fed AS supplemented diet (diet 3) no induction of the specific enzyme, ASS-ase, was found after 28 d of the experiment in comparison to other fed groups.

Discussion

The first question to be asked concerns the reliability of the ascorbate analysis used in the present study in comparison to the common techniques using DNPH. In the original description of ascorbic acid analysis (Carr et al. 1983), only the reduced form was measured, which neglected the dehydroascorbic acid (assuming that diketogulonic acid only occurs in trace amounts in animal tissues). If the reduced and oxidized ascorbate are readily interchangeable in vivo due to the enzymatic and nonenzymatic reactions (Basu et al. 1979; present work), this assay will highly underestimate the total ascorbate pool (Thomas et al. 1985). However, if the DNPH method is used directly to measure total ascorbate after the DCIP oxidation step (Tucker and Halver 1984), the amount of ascorbate will be highly overestimated due to the interfering substances, which in certain tissues (liver, muscle) account for up to 30–70% of the absorbance at 524 nm. Furthermore, ascorbic acid is extremely vulnerable to oxidation during sample processing and unless its degradation is prevented with EDTA this will introduce another error in the method. Basically, the enzymatic method (Cowey et al. 1985) estimates the reduced form of ascorbate and involves similar pitfalls to the colorimetric method if no two step procedure is introduced in the dithiothreitol reduction of DA (Wunderling et al. 1986).

Ascorbic sulfate analysis based on acid hydrolysis of extracts in order to release AA (Tucker and Halver 1984, 1986; Dabrowski and Hinterleitner 1989) leads to the release of interfering substances. Hydrolysis accomplished with $KBr0_3$ is far more selective, but still requires correction for interfering compounds (Fig. 1).

Due to the uncertainties in ascorbate determination by other authors in which no correction for the background interferences were introduced, the comparison of the present results to the data in the literature is only fragmentary. In rainbow trout, at the end of a 20-wk feeding trial with a diet lacking ascorbic

TABLE 4. Ascorbic acid and mineral concentrations in plasma of rainbow trout after 28 d feeding period.

	Treatment			
	Fasting	Diet 1	Diet 2	Diet 3
Ascorbate[1] ($\mu g \cdot mL^{-1}$)				
AA[2]	3.07 ± 1.57^{b}	1.49 ± 0.91^{a}	30.7 ± 6.7^{c}	1.63 ± 0.48^{a}
DA	traces	traces	5.6 ± 1.8^{b}	1.01 ± 0.41^{a}
AS	N.D.	N.D.	N.D.	2.56 ± 1.18
Cu ($\mu g \cdot mL^{-1}$)	0.67 ± 0.14^{ab}	0.66 ± 0.06^{b}	0.78 ± 0.16^{b}	0.53 ± 0.05^{a}
Zn ($\mu g \cdot mL^{-1}$)	12.0 ± 3.2^{a}	13.9 ± 2.6^{a}	14.7 ± 3.5^{a}	12.4 ± 1.6^{a}

[1] Mean ± SD; $n=4$.

[2] Groups with common superscript letters are not significantly different ($P<0.05$).

TABLE 5. Total ascorbic acid (AA), ascorbic sulfate (AS) and dehydroascorbic acid (DA) concentrations in tissues of rainbow trout after 28 d of consuming the test diets.

Treatment		Erythrocytes	Kidney	Liver	Pyloric caeca	Spleen	Brain
				($\mu g \cdot g^{-1}$ wet tissue)			
Fasting	AA[2]	3.11 ± 0.89^{b}	74.0 ± 12.2^{b}	53.8 ± 16.7^{b}	38.7 ± 9.1^{b}	85.8 ± 6.6^{b}	148.7 ± 17.8^{a}
	DA[2]	3.01 ± 0.92	70.4 ± 11.7	21.8 ± 8.1	14.6 ± 6.6	68.9 ± 9.9	45.1 ± 6.1
	(%)[3]	N.D.[4]	4.9	59.5	62.3	19.7	69.7
Diet 1	AA	1.01 ± 0.30^{a}	34.2 ± 9.6^{a}	22.5 ± 4.6^{a}	15.2 ± 2.9^{a}	61.7 ± 14.4^{a}	144.6 ± 22.1^{a}
	DA	1.03 ± 0.21	32.8 ± 10.2	7.1 ± 2.4	4.4 ± 1.1	41.1 ± 15.2	43.8 ± 4.2
	(%)	N.D.	4.1	68.5	71.1	23.4	69.8
Diet 2	AA	15.01 ± 4.8^{c}	219.3 ± 43.2^{c}	151.6 ± 32.5^{c}	223.0 ± 59.4^{c}	254.3 ± 85.0^{c}	211.1 ± 44.0^{b}
	DA	14.86 ± 3.3	111.7 ± 11.4	21.7 ± 5.4	27.5 ± 12.8	163.1 ± 21.3	43.3 ± 8.4
	(%)	N.D.	49.1	85.7	87.7	35.9	79.5
Diet 3	AA	3.09 ± 0.81^{b}	63.4 ± 12.4^{b}	30.2 ± 12.2^{ab}	30.9 ± 13.1^{b}	78.3 ± 15.6^{ab}	155.3 ± 27.8^{a}
	DA	3.27 ± 0.40	53.7 ± 7.8	12.8 ± 4.5	6.0 ± 3.3	70.6 ± 11.6	45.4 ± 7.8
	(%)	N.D.	15.3	57.7	80.6	9.8	70.7
	AS	N.D.	6.04 ± 2.95	5.19 ± 2.91	12.06 ± 4.5	N.D.	N.D.

[1] Groups with common superscript letters within a column are not significantly different ($P<0.05$).

[2] Mean ± SD; $n=4$.

[3] Percentage indicates proportion of the reduced form.

[4] N.D. = not detectable.

acid, the total ascorbic acid in the liver was 21 $\mu g \cdot g^{-1}$, which might indicate, taking into account the body (and liver) weight gain, that AA had been synthesized (Sato et al. 1983). In the same experiment, trout which were offered a diet with 200 mg $AA \cdot kg^{-1}$ had a hepatic ascorbate level of 277 $\mu g \cdot g^{-1}$ (Sato et al. 1983). With graded levels of dietary ascorbate (40–1280 $mg \cdot kg^{-1}$), total AA in the kidney was shown to increase gradually from 12 to 144 $\mu g \cdot g^{-1}$ (Hilton et al. 1978). The discrepancy between our results, where 337 $mg \cdot kg^{-1}$ of total dietary AA level (Dabrowski and Kock 1989) resulted in kidney AA of 219 $\mu g \cdot g^{-1}$ within 28 d, and the data mentioned above (Hilton et al. 1978), may be due to the considerable loss of supplemented AA during diet processing, which was not taken into account by the other authors (Sato et al. 1983; Hilton et al. 1978).

Diet 3 contained 357.3 $mg \cdot kg^{-1}$ of AA in the form of AS (Dabrowski and Kock 1989). However, the separate assay run on an AS preparation that had been stored at −18°C for several months, revealed that approximately 2% of AS was already hydrolysed to AA. Further studies on AS hydrolysis in the stored diet proved that 2.5–3% of AA is released during the prolonged (several months) storage. This amount, in the diet supplemented with high AS level (1084 $mg \cdot kg^{-1}$) could play a significant role in the noticeable increase in tissue AA level of fish fed diet 3 (Table 5) in comparison to the control group (diet 1). Studies carried out simultaneously (Dabrowski and Kock 1989) indicated that dietary AS concentrated in the digestive tract content of rainbow trout, and the majority was excreted via the feaces. The presence of AS in several tissues of trout fed an AS-containing diet indicated, however, that small amounts of this compound were absorbed in the gastrointestinal tract and transported in blood to liver. However, utilization of this compound via enzymatic hydrolysis seemed to be negligible (Table 5).

In trout fed the diets containing 1760 mg of $AA \cdot kg^{-1}$ diet in the form of AS or AA, the level of AA in liver was considerably lower in the former (177 $\mu g \cdot g^{-1}$) than in the latter group (271 $\mu g \cdot g^{-1}$) (Tsujimura et al. 1978). It is hypothesized that the antiscorbutic activity of AS in fish (normal growth, no external vitamin C deficiency symptoms) (Halver et al. 1975; Tsujimura et al. 1978) is due to the traces of AA or other decomposition product(s) (Shapiro and Poon 1975) formed by chemical hydrolysis of AS during food processing and prolonged storage. Prolonged feeding with AS supplemented diet leads to severe depletion of tissue ascorbate in trout (Tsujimura et al. 1978) and other fish, catfish (Murai et al. 1978) and tilapia (Soliman et al. 1986).

TABLE 6. Glutathione concentrations in tissues of rainbow trout.

Treatment	Plasma	Erythrocytes	Kidney	Liver	Pyloric caeca	Spleen	Brain
	(μM·g^{-1} wet weight of tissue and nM·mL^{-1} of plasma)						
Initial	$58.04 \pm 9.0^{1,ab}$	2.62 ± 0.33^{a}	1.34 ± 0.24^{a}	4.07 ± 0.41^{ab}	N.A.[2]	N.A.	0.96 ± 0.28^{a}
Fasting	66.9 ± 18.8^{ab}	2.56 ± 0.70^{a}	2.27 ± 0.35^{b}	3.56 ± 0.33^{a}	3.41 ± 0.60^{a}	3.26 ± 0.58^{a}	1.60 ± 0.22^{b}
Diet 1	43.8 ± 18.0^{a}	2.97 ± 0.82^{a}	2.90 ± 0.34^{c}	4.43 ± 0.31^{b}	3.32 ± 0.68^{a}	3.84 ± 0.21^{ab}	1.51 ± 0.08^{b}
Diet 2	72.0 ± 18.9^{b}	3.32 ± 0.34^{a}	2.94 ± 0.38^{c}	4.39 ± 0.62^{b}	3.21 ± 0.47^{a}	4.00 ± 0.56^{b}	1.87 ± 0.68^{ab}
Diet 3	84.2 ± 36.6^{b}	2.58 ± 0.37^{a}	2.88 ± 0.29^{c}	4.22 ± 0.19^{b}	3.74 ± 0.08^{a}	3.87 ± 0.43^{ab}	1.58 ± 0.11^{b}

[1] Results are expressed as means ± SD; $n = 4$–5; within a column values not sharing a common superscript letter are significantly different ($P < 0.05$).

[2] N.A. = Not analyzed.

TABLE 7. Activities of enzymes of rainbow trout after termination of the experiment.

	Tissue	Dietary treatment			
		Fasting	Diet 1	Diet 2	Diet 3
Dehydroascorbate reductase (μM AA·mg $protein^{-1}$·h^{-1})	Liver	$135 \pm 50^{1,a}$	247 ± 158^{ab}	617 ± 322^{b}	334 ± 341^{ab}
	Kidney	N.D.	N.D.	125 ± 86^{a}	58 ± 23^{a}
	Pyloric caecae	N.D.	N.D.	N.D.	N.D.
Arysulfatase (AR-ase) (μM NCS·mg $protein^{-1}$·h^{-1})	Liver	36.3 ± 16.0^{b}	20.5 ± 8.0^{ab}	16.6 ± 6.6^{a}	22.2 ± 13.3^{ab}
	Pyloric caecae	46.6 ± 26.2^{a}	54.3 ± 29.2^{a}	54.5 ± 29.8^{a}	35.0 ± 12.1^{a}
Ascorbic sulfate sulfohydrolase (ASS-ase) (μg AA·mg $protein^{-1}$·h^{-1})	Liver	2.4 ± 0.8^{a}	10.8 ± 7.4^{b}	8.8 ± 5.8^{b}	5.8 ± 1.0^{b}
	Pyloric caecae	N.D.	N.D.	N.D.	N.D.

[1] Results are expressed as means ± SD; $n = 4$; within a line values not sharing a common superscript letter are significantly different ($P < 0.05$).

[2] N.D. = Not detectable.

In the scurvy-prone rat mutant an increase of dietary ascorbate from 0 to 300 mg·kg^{-1} diet led to the gradual increase of liver AA from approximately 15–145 μg·g^{-1} (Horio et al. 1985), whereas in mice, an animal capable of endogenously synthesizing AA, the megadose increase of dietary vitamin C (0.076 to 8%) only slightly changed the AA levels in liver and kidney, i.e. from 275 to 473 and from 214 to 650 μg·g^{-1}, respectively (Tsao et al. 1987). This suggests that in mice the exogenous AA uptake influenced ascorbate synthesis, whereas in scurvy-prone animals, including fish, an increase in dietary AA level enhanced tissue accumulation (Hilton et al. 1978; Horio et al. 1985). However, the most recent studies on the absorption of AA suggest that, in humans, both gastrointestinal absorption and reabsorption in the kidney are saturable processes (Melethil et al. 1986). There is also evidence that in guinea pigs fed increasing amounts of AA the liver AA level tends to saturate at the dietary level of 1% AA, whereas adrenal AA was already saturated at 0.1% AA (Ginter et al. 1979).

AS is a natural form of ascorbate which has been reported to occur in the urine of humans (Baker et al. 1971), tissues of rat (Mumma and Verlangieri 1972), and dormant cysts of the invertebrate *Artemia salina* (Dabrowski and Hinterleitner 1989). However, determination of AA in the urine after oral administration of megadoses of AS (5–7.5 g per capita) revealed no hydrolysis of AS and only 2.1–2.5% of the AS was excreted in the urine (Tsujimura et al. 1982). It seems that orally administered AS is excreted in feces both in humans (Tsujimura et al. 1982) and in salmonid fish (Dabrowski and Kock 1989).

In the guinea pig, increasing the dose of AS to 16-fold the AA requirement level does not prevent scurvy (Kuenzig et al. 1974). The ascorbate level in the liver of guinea pigs fed an AS-supplemented diet already fell to 26 μg·g^{-1} after 5 d, in comparison to 65 μg·g^{-1} in the liver of animals fed an AA diet (Tsujimura et al. 1976). It is therefore reasonable to conclude that the salmonid fish and scurvy-prone mammal do not differ in their handling of orally administered AS. The lack of vitamin C deficiency symptoms in trout receiving large doses of AS seems to be due to their poikilothermy and lower metabolic rate. The prolonged experiments on fish (3–5 mo) apparently led to the release of AS decomposition products (Shapiro and Poon 1975) which include AA in its available form, thus satisfactorily preventing scurvy. In small mammals, guinea pig and monkey, the dramatic decrease in tissue AA level took place within a week (Tsujimura et al. 1976) and was followed by external signs of vitamin C deficiency.

AR-ase activity has been reported in several mammals (Waheed et al. 1985) including guinea pig (Leville and Schwartz 1982). AR-ase activity in adrenals of guinea pig fed a vitamin C deficient diet showed a 50% increase as compared to animals fed a high level of ascorbate (Leville and Schwartz 1982). This might have been due to growth depression, resulting in fasting induced by ascorbate deficiency, which led to the decrease in collagen and proteoglycan synthesis (Bird et al. 1986). The present study, in which AR-ase activity was highest in fasting fish, and was higher in fish fed ascorbic acid depleted diets (diet 1 and 3) than in fish receiving ascorbic acid, corroborated the findings in guinea pig.

ASS-ase activity in bovine and rat hepatic tissue have been reported to be 2.43 (Carlson et al. 1977) and 3.52 μg AA mg $protein^{-1}$·h^{-1} (Thompson and Daniel 1987), respectively, and the values found in the present work on salmonid fish (Table 7) fall within the same range. Previously reported ASS-ase activity in crude extracts of trout liver amounted to 1.46 μg AA mg protein$^{-1}\cdot$h^{-1} (Benitez and Halver 1982), but in such assays no correction was made for DCIP color change due to the enzyme addition itself. Also, as reported for bovine ASS-ase (Carlson et al. 1977), rainbow trout ASS-ase is extremely sensitive to phosphate inhibition (unpubl. data) and dialysis of the enzyme extract is essential.

Under the conditions listed in Methods, DA-ase yielded the highest rate of activity and the concentrations of GSH and DA were higher than in the original description for mammalian tissue (Stahl et al. 1983). However, the rates of enzymatic and nonenzymatic reduction of dehydroascorbate obtained in the present study were similar to those in human blood cells (Stocker et al. 1986). Other reported values for fish DA-ase suggested activities of 186 and 45 nM mg protein$^{-1}\cdot$h^{-1}, respectively, in liver and kidney of rainbow trout (Yamamoto et al. 1977). In guinea pig and rat, DA-ase activity was reported in all tissue examined. It was also proven by DA injection that the reduction was far more efficient in the guinea pig than the rat (Tsujimura et al. 1983). Within 60 min of injection of DA, the concentration of reduced ascorbate was increased 16-fold in guinea pig liver, but only 1.6-fold in rat liver. Other systems of DA reduction must operate in both fish and higher vertebrates. Results obtained suggest, however, that in the intestinal tissue, 62–87% of the ascorbate was in the reduced form (Table 5). At the same time, we were unable to detect DA-ase activity in the intestine of rainbow trout.

At concentrations of 10–100 μM, GSH provides protection against AA oxidation (Winkler 1987). In the present study 58 μM were found in fish plasma. In human plasma higher levels have been reported, i.e. 311–556 μM (Wayner et al. 1987). However, in the in vivo system, the SH-group and ascorbate contribute only partially to the antioxidant system (Thomas and Wofforsd 1984). The GSH concentration in fish tissues (Table 6) is far higher than that used in an in vitro system (Winkler 1987), but still it does not protect against ascorbic acid oxidation. In fish tissues, liver in particular, GSH is the dominant sulfhydryl substance (Thomas and Wofforsd 1984). The present results therefore warrant further studies in which other components of the antioxidant system (ureate – Wayner et al. 1987) should be identified and the GSH regenerating system tested.

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References

Baker, E. M., D. C. Hammer, S. C. March, B. M. Tolbert, and J. E. Canham. 1971. Ascorbate sulfate: a urinary metabolite of ascorbic acid in man. Science (Wash., DC) 173: 826–827.

Basu, S., S. Som, S. Deb, and D. Mukherjee. 1979. Dehydroascorbic acid reduction in human erythrocytes. Biochem. Biophys. Res. Commun. 90: 1335–1340.

Benitez, L. V., and J. E. Halver. 1982. Ascorbic acid sulfate sulfohydrolase (C_2sulfatase): the modulator of cellular levels of ascorbic acid in rainbow trout. Proc. Natl. Acad. Sci. USA 70: 5445–5449.

Bird, R. A., R. G. Spanheimer, and B. Peterkofsky. 1986. Coordinate regulation of collagen and proteoglycan synthesis in costal cartilage of scorbutic and acutely fasted vitamin C-supplemented guinea pigs. Arch. Biochem. Biophys. 246: 42–51.

Carlson, R. W., M. Downing, and B. M. Tolbert. 1977. Purification and kinetic and physical characterization of bovine ascorbate-2-sulfate sulfhydrolase. Biochemistry 16: 5221–5227.

Carr, S., M. Bally, P. Thomas, and J. M. Neff. 1983. Comparison of methods for determination of ascorbic acid in animal tissues. Anal. Chem. 55: 1229–1232.

Chatterje, I. B. 1973. Evolution and the biosynthesis of ascorbic acid. Science (Wash., DC) 182: 1271–1272.

Cowey, C. B., J. G. Bell, D. Knox, A. Fraser, and A. Youngson. 1985. Lipids and lipid antioxidant systems in developing eggs of Atlantic salmon (*Salmo salar*). Lipids 20: 567–572.

Dabrowski, K., and S. Hinterleitner. 1989. Applications of a simultaneous assay of ascorbic acid, dehydroascorbic acid and ascorbic sulfate in biological material. Analyst 114: 83–87.

Dabrowski, K., and G. Kock. 1989. Absorption of ascorbic acid and ascorbic sulfate and their interaction with minerals in digestive tract of rainbow trout. Can. J. Fish. Aquat. Sci. 46: 1952–1957.

Daniel, W. L., L. Leonard, and T. Carmody. 1987. Comparative biochemistry of fish and amphibian arysulfatases. Comp. Biochem. Physiol. 87B: 385–390.

Ginter, E., P. Bobek, and D. Vargova. 1979. Tissue levels and optimum dosage of vitamin C in guinea pigs. Nutr. Metab. 23: 217–226.

Halver, J. E. 1985. Recent status in vitamin nutrition and metabolism in fish. p. 415–429. *In* C. B. Cowey, A. Mackie, and J. G. Bell [ed.] Nutrition and Feeding in Fish. Academic Press, London, U.K.

Halver, J. E., R. R. Smith, B. M. Tolbert, and E. M. Baker. 1975. Utilization of ascorbic acid in fish. Ann. New York Acad. Sci. 258: 81–102.

Hilton, J. W., C. Y. Cho, and S. J. Slinger. 1978. Effect of graded levels of supplemented ascorbic acid in practical diets fed to rainbow trout. J. Fish. Res. Board Can. 35: 431–436.

Horio, F., K. Ozaki, A. Yoshida, S. Makino, and Y. Hayashi. 1985. Requirement for ascorbic acid in a rat mutant unable to synthesize ascorbic acid. J. Nutr. 115: 1630–1640.

Hughes, S. G., R. C. Riis, J. G. Nickum, and G. L. Rumsey. 1981. Biomicroscopic and histologic pathology of the eye in riboflavin deficient rainbow trout. Cornell Vet. 71: 269–279.

Kuenzig, W., R. Avenia, and J. J. Kamm. 1974. Studies on the antiscorbutic activity of ascorbate 2-sulfate in the guinea pig. J. Nutr. 104: 952–956.

Leville, C. R., and E. R. Schwartz. 1982. Effect of ascorbate on lysosomal enzyme activities in guinea pig cartilage and adrenals. Int. J. Vitam. Nutr. Res. 52: 436–441.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.

Machlin, L. J., F. Garcia, W. Kuenzig, C. B. Richter, H. E. Spiegel, and M. Brin. 1976. Lack of antiscorbutic activity of ascorbate 2-sulfate in the rhesus monkey. Am. J. Clin. Nutr. 28: 825–831.

Melethil, S., W. D. Mason, and C. J. Chang. 1986. Dose–dependent absorption and excretion of vitamin C in humans. Int. J. Pharm. 31: 83–89.

Mumma, R. O., E. E. McKee, A. J. Verlangieri, and G. P. Barron. 1972. Antiscorbutic effect of ascorbic acid 2-sulfate in the guinea pig. Nutr. Rep. Int. 6: 133–137.

Mumma, R. O., and A. J. Verlangieri. 1972. Isolation of ascorbic acid 2-sulfate from selected rat organs. Biochem. Biophys. Acta. 273: 249–253.

Murai, T., J. W. Andrews, and J. C. Bauernfeind. 1978. Use of L-ascorbic acid, ethocel coated ascorbic acid and ascorbic 2-sulfate in diets of channel catfish (*Ictalurus punctatus*). J. Nutr. 108: 1761–1766.

Nimmo, I. A. 1985. The glutathione S-transferase activity in the gills of rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 80B: 365–369.

Sato, M., T. Kondo, R. Yoshinaka, and S. Ikeda. 1983. Effect of water temperature on the skeletal deformity in ascorbic acid deficient rainbow trout. Bull. Jpn. Soc. Sci. Fish. 49: 443–446.

Shapiro, S. S., and J. P. Poon. 1975. Apparent sulfation of glycosoaminoglycans by ascorbic acid 2-/^{35}S/ sulfate: an explanation. Biochem. Biophys. Acta. 385: 221–231.

Soliman, A. K., K. Jauncey, and R. J. Roberts. 1986. The effect of varying forms of dietary ascorbic acid on the nutrition of juvenile tilapia (*Oreochromis niloticus*). Aquaculture 59: 197–208.

Stahl, R. L., L. F. Liebes, C. M. Farber, and R. Silber. 1983. A spectrophotometric assay for dehydroascorbate reductase. Anal. Biochem. 131: 341–344.

Stocker, R., M. J. Weidemann, and N. H. Hunt. 1986. Possible mechanisms responsible for the increased ascorbic acid content of *Plasmodium vinckei*-infected mouse erythrocytes. Biochem. Biophys. Acta. 881: 391–397.

Terada, M., Y. Watanabe, M. Kunitomo, and E. Hayashi. 1978. Differential rapid analysis of ascorbic acid and ascorbic 2-sulfate by dinitrophenylhydrazine method. Anal. Biochem. 84: 604–606.

Thomas, P., M. B. Balley, and J. M. Neff. 1985. Influence of some environmental variables on the ascorbic acid status of mullet, *Mugil cephalus* L., tissues. II. Seasonal fluctuations and biosynthetic ability. J. Fish. Biol. 27: 47–57.

Thomas, P., and H. W. Wofforsd. 1984. Effects of metals and organic compounds on hepatic glutathione, cysteine, and acid-soluble thiol levels in mullet (*Mugil cephalus* L.). Toxicol. Appl. Pharmacol. 76: 172–182.

Thompson, D. B., and W. L. Daniel. 1987. Isolation and characterization of rat hepatic ascorbic acid-2-sulfatases. Enzyme (Basel) 37: 134–140.

Tsao, C. S., P. Y. Leung, and M. Young. 1987. Effect of dietary ascorbic acid intake on tissue vitamin C in mice. J. Nutr. 117: 291–297.

Tsujimura, M., T. Fukuda, and T. Kasai. 1983. On the reduction of dehydroascorbic acid in guinea pig and rat. Nutr. Rep. Int. 28: 881–890.

1982. Studies on the excretion of ascorbic acid 2-sulfate and total vitamin C into human urine after oral administration of ascorbic acid 2-sulfate. J. Nutr. Sci. Vitaminol. 28: 467–476.

Tsujimura, M., H. Yoshikawa, T. Hasegawa, and T. Suzuki. 1976. Antiscorbutic activity of ascorbic acid 2-sulfate in the guinea pig. Vitamins (Kyoto) 50: 19–25.

Tsujimura, M., H. Yoshinaka, T. Hasegawa, T. Suzuki, T. Kasai, T. Suwa, and S. Kitamura. 1978. Studies on the vitamin C activity of ascorbic acid 2-sulfate on the feeding test of new born rainbow trout. Vitamins (Kyoto) 52: 35–44.

Tucker, B. W., and J. E. Halver. 1984. Distribution of ascorbate-2-sulfate and distribution, half life and turnover rate of /1-^{14}C/ascorbic acid in rainbow trout. J. Nutr. 114: 991–1000.

1986. Utilization of ascorbate-2-sulfate in fish. Fish. Physiol. Biochem. 2: 151–160.

Waheed, A., J. M. Risley, and R. L. Van Etten. 1985. Structural and immunological relationships among mammalian arylsulfatase A enzymes. Comp. Biochem. Physiol. 82B: 855–862.

Wayner, D. D. M., G. W. Burton, K. U. Ingold, and L. R. C. Barclay. 1987. The relative contribution of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochem. Biophys. Acta. 924: 408–419.

Winkler, B. S. 1987. In vitro oxidation of ascorbic acid and its prevention by GSH. Biochem. Biophys. Acta. 925: 258–264.

Wunderling, M., H.-H Paul, and W. Lohmann. 1986. Evaluation of a direct spectrophotometric method for the rapid determination of ascorbate and dehydroascorbate in blood using ascorbate oxidase. Biol. Chem. Hoppe-Seyler 367: 1047–1054.

Yamamoto, Y., M. Sato, and S. Ikeda. 1977. Biochemical studies on L-ascorbic acid in aquatic animals-VII. Reduction of dehydro-L-ascorbic acid in fishes. Bull. Jpn. Soc. Sci. Fish. 43: 53–57.

1978. Existence of L-gulonolactone oxidase in some teleosts. Bull. Jpn. Soc. Sci. Fish. 44: 775–779.

Distribution and Growth of Pre-Recruit Cohorts of the Northern Shrimp (*Pandalus borealis*) in the Western Gulf of St. Lawrence as Related to Hydrographic Conditions[1]

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Samples collected with a suprabenthic sled from May to October were analysed to determine the number of pre-recruit cohorts in the northern shrimp population of the Gulf of St. Lawrence and to estimate their growth and distribution. An unexpected cohort was found with carapace lengths of 4.5–9.5 mm. This appeared to be cohort I, thus indicating three pre-recruit cohorts: cohorts 0 (2.5–4.5 mm), I (4.5–9.5 mm), and II (9.5–14.5 mm). The comparatively slow growth rate of 4–5 mm per year was attributed to the low temperatures (0.5–2.0°C) in the bottom water inhabited by the pre-recruits, immediately below the intermediate cold water mass of the Gulf of St. Lawrence. Growth rates peaked in August–September, reaching 1 mm per month. Benthic settlement of the postlarvae also appeared to occur in August–September, at an age of 4–5 mo. Sexual differentiation in males started at an age of 16 mo and maturity was reached by 30–37 mo. Two strata were sampled, 0.3–0.7 m and 1.0–1.4 m above the bottom. More than 70% of the shrimps were concentrated in the first 0.3–0.7 m stratum. Maximum density observed for one single cohort was 67 individuals per 100 m^2. Large spatial and temporal variations of densities occured daily, semimonthly, and seasonally, and are thought to be partially related to horizontal transport of water masses. Densities in the strata sampled were significantly lower at night, as a result of vertical migrations in the water column.

Une collection d'échantillons prélevés au moyen d'un traineau suprabenthique de mai à octobre est analysée pour déterminer le nombre de cohortes pré-recrutées chez la population de crevette nordique du golfe du Saint-Laurent et pour estimer leur croissance et leur répartition spatiale. Une cohorte insoupçonnée a été mise en évidence aux longueurs de carapace de 4.5–9.5 mm. Cette cohorte s'est avérée être la cohorte I, ce qui indique trois cohortes pré-recrutées, soit : les cohorte 0 (2.5–4.5 mm), I (4.5–9.5 mm), et II (9.5–14.5 mm). Le faible taux de croissance de 4–5 mm par an est attribué aux basses températures (0.5–2°C) de la masse d'eau sur les fonds habités par les pré-recrues, situés immédiatement sous la nappe intermédiaire glaciale du golfe du Saint-Laurent. Les taux de croissance atteignaient des sommets de 1 mm par mois en août–septembre. L'installation benthique des post-larves a semblé survenir en août–septembre, à l'âge de 4–5 mois. La différenciation sexuelle en mâles commençait à l'âge de 16 mo et leur maturité était atteinte à l'âge de 30–37 mo. Deux strates furent échantillonnées, 0.3–0.7 m et 1.0–1.4 m à partir du fond. Plus de 70% des crevettes étaient concentrées dans la strate de 0.3–0.7 m. La densité maximale observée pour une seule cohorte a été de 67 individus par 100 m^2. D'importantes variations de densité ont été observées aux échelles quotidienne, semi-mensuelle et saisonnière, et ont semblé dépendre en partie du transport horizontal des masses d'eau. Les abondances dans les strates échantillonnées étaient significativement plus faibles pendant la nuit, par suite de migrations verticales dans la colonne d'eau.

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The northern shrimp (*Pandalus borealis*) is the object of important fisheries in northern countries throughout the world. Commercial catches are obtained from soft mud bottoms of 50–500 m in depth by means of bottom trawls. Because it is primarily the largest individuals that are sought by the fishery and easily collected by the fishing trawls, most studies have been directed at the later developmental stages. Pre-recruit shrimp, smaller than the 10% trawl selectivity length of 15 mm carapace length (CL) (Labonté et Fréchette 1978), have almost always been neglected in field population studies and knowledge of their ecology is still poor (see review by Shumway et al. 1985), despite the important role they play in stock dynamics. The present paper is a contribution to fill this gap.

Throughout its latitudinal distribution range, this protandric hermaphrodite species varies widely in growth rate, age at maturity, age at sex reversal, and duration of ovigerous period

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[1] Contribution to the programs of GIROQ (Groupe interuniversitaire de recherches océanographiques du Québec) and of the Division de la Recherche sur les Pêches de l'Institut Maurice-Lamontagne.

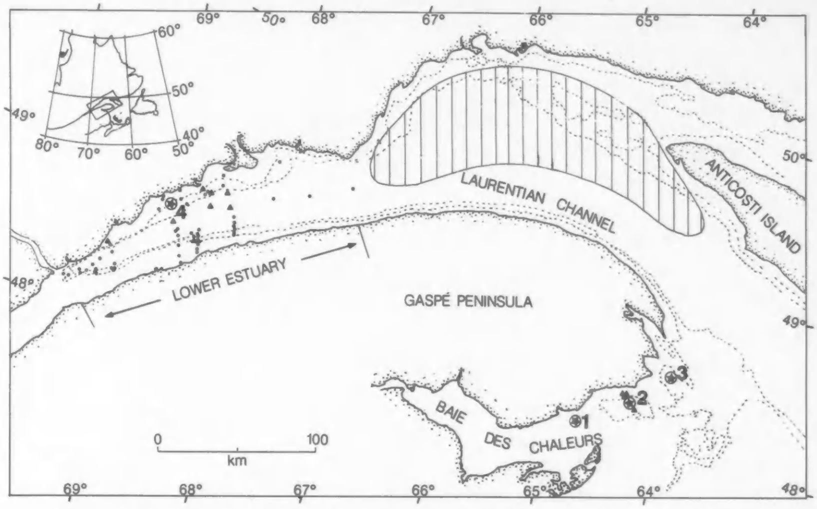

FIG. 1. Map of the western Gulf of St. Lawrence showing bathymetry (100 m and 200 m contours) and positions of sampling stations. Stars: monitoring stations; circles: singly sampled stations; triangles: sampled twice or more. Hatched area: Sept-Iles adult northern shrimp stock.

TABLE 1. Mean oblique carapace lengths (OCL in mm, SD in parentheses) and monthly increments of OCL calculated between consecutive observations of pre-recruit *Pandalus borealis* in the western Gulf of St. Lawrence, from ages 0 (= 15 April) to 2.5 yr.

		OCL		Monthly Increment	
Age (yr)	Date	Estuary	Baie des Chaleurs	Estuary	Baie des Chaleurs
0.318	Aug.		3.35 (0.42)		
0.359	Aug.		3.42 (0.26)		0.13
0.381	Sept.		3.47 (0.26)		0.19
0.410	Sept.		3.55 (0.29)		0.23
0.447	Sept.		3.53 (0.36)		< 0
1.121	May	6.42 (0.54)	6.10 (0.65)		(0.31)
1.156	May		6.42 (0.50)		0.75
1.159	June	6.42 (0.54)		0.00	
1.197	June		6.70 (0.50)		0.56
1.236	June		6.76 (0.52)		0.13
1.241	July	6.78 (0.59)		0.36	
1.274	July	6.98 (0.66)	7.08 (0.60)	0.50	0.69
1.318	Aug.		7.60 (0.67)		0.97
1.329	Aug.	7.25 (0.71)		0.40	
1.353	Aug.	7.54 (0.69)		0.99	
1.359	Aug.		7.64 (0.56)		0.08
1.381	Sept.	7.89 (0.77)	7.77 (0.63)	1.03	0.49
1.410	Sept.		8.26 (0.75)		1.39
1.441	Sept.	8.15 (0.72)		0.36	
1.447	Sept.		8.24 (0.63)		< 0
1.482	Oct.		8.47 (0.59)		0.54
1.496	Oct.	8.28 (0.59)		0.19	
1.515	Oct.		8.50 (0.64)		0.07
2.121	May	10.87 (0.94)		(0.34)	
2.159	June	10.93 (0.83)		0.13	
2.241	July	11.31 (1.09)		0.38	
2.274	July	11.42 (1.05)		0.27	
2.329	Aug.	11.78 (1.10)		0.54	
2.353	Aug.	12.15 (0.99)		1.27	
2.381	Sept.	12.44 (1.20)		0.85	
2.441	Sept.	12.26 (1.06)		< 0	
2.496	Oct.	12.63 (0.95)		0.55	

TABLE 2. Percentage (with 95% C.I.) of *Pandalus borealis* caught in the lower (0.3–0.7 m above bottom) and upper (1.0–1.4 m above bottom) nets of the suprabenthic sled at monitoring stations 2, 3, and 4.

	Day		Night	
Stations	Lower	Upper	Lower	Upper
Baie des Chaleurs				
Stn. 2. Cohort I	74 (68–79)	26 (21–32)	(Insufficient numbers)	
Stn. 3 I	93 (91–95)	7 (5–9)	- - - -	- - - -
Estuary				
Stn. 4. Cohort I	98 (97–99)	2 (1–3)	90 (86–93)	10 (7–14)
II	97 (97–99)	3 (1–3)	96 (96–98)	4 (2–4)
>II	88 (84–91)	12 (9–16)	86 (80–91)	14 (9–20)

(Rasmussen 1953; Horsted and Smidt 1956; Allen 1959). Though northern shrimp of cohort I in most populations are thought to be larger than 10 mm CL (Shumway et al. 1985), recent observations of cod stomach contents on shrimp grounds off Newfoundland–Labrador suggest that they are much smaller, from 5 to 10 mm CL (Parsons et al. 1986). Thus, the mode of length–frequency distributions generally found between 10–15 mm CL may correspond to cohort II rather than cohort I. Proper identification of the first cohorts is of fundamental importance for determination of the ages represented by modes on length–frequency distributions (e.g. Macdonald and Pitcher 1979) which is required for population dynamics modelling. This problem initiated the present study which is a first look at growth and distribution of early cohorts in the Gulf of St. Lawrence, from preserved samples taken with a suprabenthic sled. Specific objectives were (1) to investigate the presence of a first cohort at carapace lengths smaller than 10 mm, (2) to estimate the time of benthic settlement of the larvae and measure the local growth rates of pre-recruits and, (3) to estimate temporal and spatial variations of pre-recruit density on the bottom.

TABLE 3. Percentage (with 95% C.I.) of individuals bearing the characters of the next developmental stages for cohorts I (> juvenile stage) and II (> immature male). Insufficient numbers are indicated by dashes. * = Data from 4 neighbouring stations.

	Estuary 1973 Station 4		Baie des Chaleurs 1969 Station 3	Station 2
Date	Cohort I	Cohort II	Cohort I	Cohort II
May 29–30	0 (0–1)	15 (11–20)	0 (0–6)	0 (0–26)
June 11–13	0 (0–1)	12 (9–15)	1 (0–4)	—
25–27			1 (0–3)	0 (0–10)
July 9–13	0 (0–3)	19 (14–25)	0 (0–3)	—
23–25	0 (0–3)	15 (10–21)	10 (4–20)	9 (0–45)
August 6–8			13 (3–31)	0 (0–10)
13–14	0 (0–3)	28 (21–36)		
22–28	11 (4–23)*	61 (51–71)*	25 (15–38)	—
September 2–3	25 (8–55)	45 (28–66)		20 (15–28)
11–12			47 (36–58)	6 (1–20)
24–25	22 (12–35)	42 (32–52)	34 (24–45)	19 (4–48)
October 7			78 (58–90)	33 (20–48)
13	18 (11–27)	51 (43–57)		
20			46 (32–61)	20 (11–32)

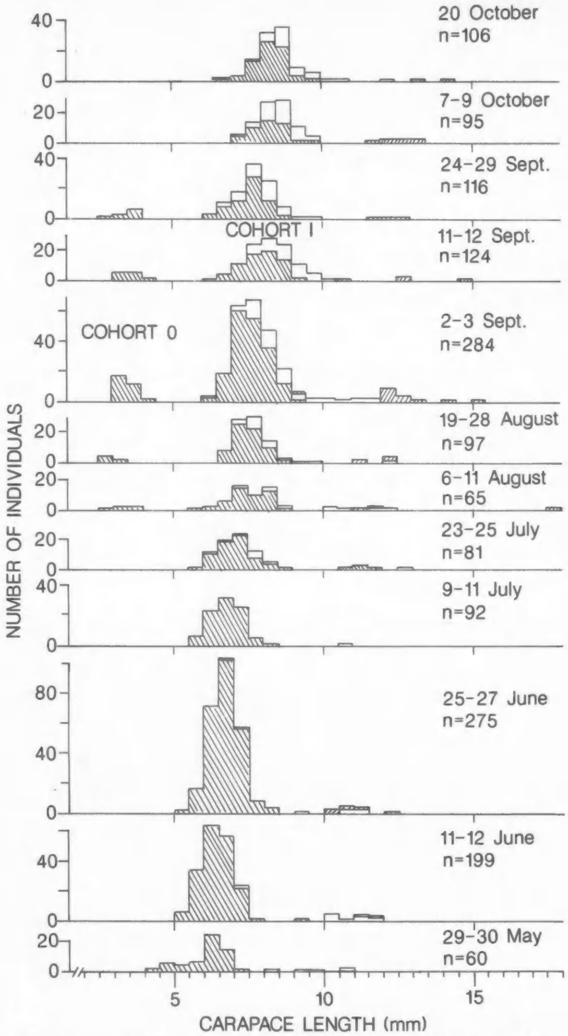

FIG. 2. Length–frequency distribution of pre-recruits of *Pandalus borealis* in Baie des Chaleurs in 1969. Most (~80%) specimens were caught at outer station 3, except on 2–3 September (station 2 alone). Symbols as in Fig. 3.

Methods

Samples were collected with a suprabenthic sled (Brunel et al. 1978) carrying two 0.5 mm mesh plankton nets mounted one above the other which sampled two strata at 0.3–0.7 m and 1.0–1.4 m above the bottom. The nets were equipped with TSK flowmeters and were opened and closed by a mechanism triggered by bottom contact. The sled was towed at a speed of 2 knots for 5–35 min. Temperature profiles were obtained at monitoring stations 2 and 4 (Fig. 1) with a Wallace Tierman bathythermograph and with inverted thermometers mounted on Nansen bottles.

Sampling was carried out in the western Gulf of St. Lawrence off Baie des Chaleurs in 1969, and in the Lower St. Lawrence Estuary between 1970 and 1973 (Fig. 1). Four monitoring stations located at 70–125 m depth, on silt-clay bottoms (Poirier 1971; Loring and Nota 1973; Robert 1974), were visited semimonthly from May to October. Stations 1, 2, and 3, in the outer reaches of Baie des Chaleurs, were located respectively 110, 70, and 35 km from the 200 m contour of the Laurentian Channel and water depths of 70–90 m, 115–120 m, and 120–125 m respectively. One daylight tow was taken semimonthly at stations 1 and 3. Diurnal and nocturnal tows were taken semimonthly at station 2, except on 2–3 September, when 26 samples were collected at 30-min intervals over an 18-h cycle. At station 4 (120 m) in the Estuary, samples were taken semimonthly at 4-h intervals over 24-h cycles. Four more daytime satellite stations were occasionally sampled off Baie des Chaleurs, and a network of 61 stations, at depths of 18–380 m, was visited in the Estuary to survey spatial and bathymetric distribution.

Samples were preserved on board in 10% formalin and transfered to 70% alcohol in the laboratory. Specimens of *Pandalus borealis* were sorted and counted by sex and developmental stage according to criteria developed by Berkeley (1930), Rasmussen (1953), and Allen (1959). The criteria are: juvenile, secondary sexual characters absent; immature male, copulatory organ present and appendix masculina absent or present with or without two small setae; pre-adult male, appendix masculina with two to 10 setae; mature male, copulatory organ fully developed and more than 10 setae on appendix masculina; transitional, endopodite with pointed apex and a thin marginal copulatory organ; female, endopodite with thin and pointed apex and without male copulatory organ, appendix masculina absent. The oblique carapace length (OCL, from posterior edge of eye orbit to middorsal posterior edge of carapace) was measured to the nearest 0.1 mm under a dissecting microscope or with a vernier caliper.

The number of individuals per m^3 from the total water volume filtered in both nets was transformed to number per m^2 by multiplying by *S*/*L*, where *S* is the total area of the mouth openings of the two nets and *L* is the width of the nets. Modal char-

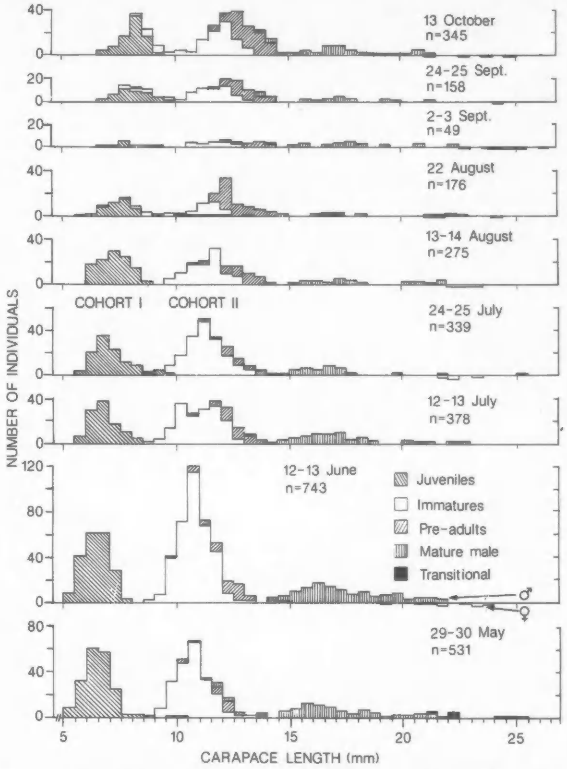

FIG. 3. Length–frequency distribution of pre-recruits of *Pandalus borealis* at station 4 in the Lower St. Lawrence Estuary in 1973. Data for August 22 come from four neighbouring stations.

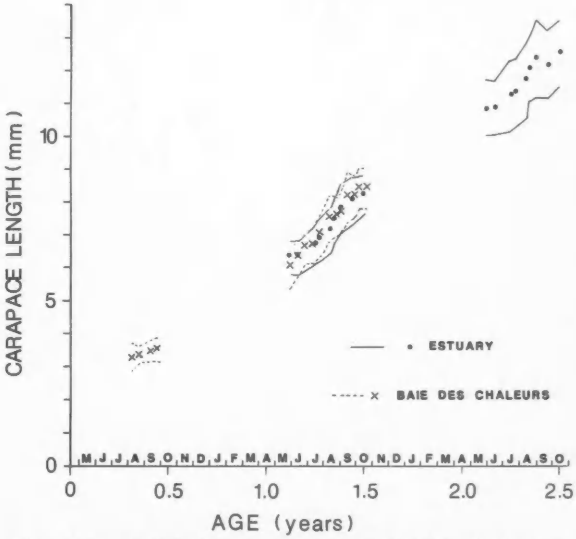

FIG. 4. Growth of *Pandalus borealis* during its first 2.5 yr in the western Gulf of St. Lawrence in 1969 (Baie des Chaleurs) and 1973 (estuary). Mean carapace length with 2-SD envelopes.

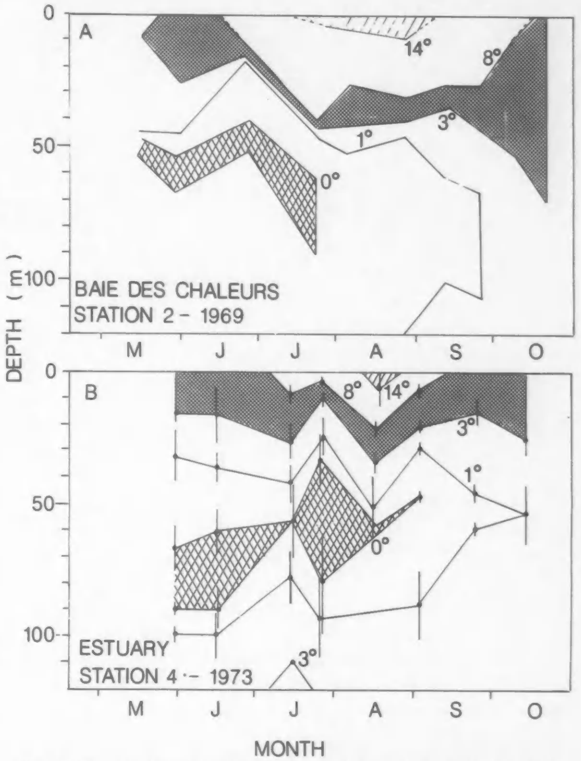

FIG. 5. Time series of isotherms (°C) at the monitoring stations 2 in Baie des Chaleurs in 1969 (A) and 4 in the Lower St. Lawrence Estuary in 1973 (B). Bars in B are the 24-h range of vertical oscillations forced by a semidiurnal internal tide. Diagram A reproduced from Poirier (1971).

acteristics of length–frequency distributions were determined according to the method of Macdonald and Pitcher (1979).

Results

Number of Pre-Recruit Cohorts and their Growth

In the two areas sampled, length–frequency distributions had three clear modes at 2.5–4.5 mm, 4.5–9.5 mm, and 9.5–14.5 mm OCL (Fig. 2 and 3). Cohorts 0, I, and II were thus easily separated as there were few overlaps (Table 1). The annual increments of carapace length of these pre-recruit cohorts were about 4.5 mm per year (Table 1). Given the standard deviations of the modes, the mean carapace length at a given age at the 2 localities and different years did not differ significantly (Table 1, Fig. 4). The increment of the mean length peaked in August–September (Table 1, Fig. 4) when the growth rate reached 1 mm per month, about three times the annual mean rate of 0.35 mm per month. About 40% of the annual growth occurred between midJune to the end of September. The largest individuals of each cohort gradually transformed into instars bearing secondary sexual characters during the burst of summer growth (Fig. 2 and 3, Table 3). The first detectable change of juveniles to immatures in cohort I occurred at the end of the summer. Half of the individuals of cohort II had reached full maturity in October, at age 30 mo, whereas all individuals of cohort III were mature in May, at age 37 mo (Fig. 3, Table 3). Bottom temperatures at the monitoring stations were low, generally from 0.5 to 2°C (Fig. 5).

Distribution and Fluctuations of Abundance

More than 70% of shrimp of all sizes were caught by the lower net of the sampler, both day and night (Table 2), which indicates a distribution close to the bottom in the suprabenthic layer sampled.

In Baie des Chaleurs, most individuals belonged to cohort I, which was relatively abundant, a few to cohort 0, and cohorts II and III were nearly absent (Fig. 2). Cohort 0 was found only in August and September, had an average density of only 0.85 per 100 m^2 (SD = 0.62, n = 19) and an average carapace length of 3.5 mm (SD = 0.3, n = 59). Cohort I density was significantly greater at the outer station 3 (Wilcoxon tests, $p<0.05$), and gradually decreased with distance from the Laurentian Channel towards the inner station 1, where it was rare (Table 4). A shift to lower densities occurred in midsummer at station 3 while the opposite was observed at the adjacent station 2. These fluctuations may reflect advection from station 3 to station 2, as they occurred in harmony with changes in water masses near the bottom at the end of the season (Fig. 5A). Freqency of occurrence of cohort I at station 1 agrees with that trend.

In the Estuary, all cohorts were most abundant near both outer slopes (100–200 m) bordering the Laurentian Channel (Fig. 6). The median depth of cohorts increased with age from 87 m for cohort I, to 111 m for cohort II, and 119 m for cohorts >II (Fig. 7), which exposed them to the low temperatures directly below the core of the intermediate cold water mass of the Gulf of St. Lawrence (Fig. 5B). The depth distribution of cohorts >II seems shallow compared to the mean depth (200–300 m) of adults in the Gulf, and may partly reflect the poor performance of our sampler in catching large shrimp. Since cohort 0 was rare, an estimation of its depth distribution was not attempted. This cohort was caught only once at four stations on the northern shelf of the Laurentian Channel, during the period from the end of August to October (Fig. 6). The abundance of all cohorts greatly fluctuated daily and throughout the season, giving standard deviations of about 100% of the corresponding means (Table 4). The average density could change semimonthly by one order of magnitude. No systematic seasonal

TABLE 4. Day (bold) and night mean (SD in parentheses) densities (no. per 100 m^2) of *Pandalus borealis* caught by the suprabenthic sled at the four monitoring stations. Significantly greater day vs. night densities are underlined (Kruskal–Wallis tests $p < 0.05$) or marked with "**" (Wilcoxon paired samples test $p < 0.05$). Dashes: no data.

	Estuary 1973 Station 4			Baie des Chaleurs 1969 Station 3 (outer)	Station 2 (middle)	Station 1 (inner)
Date	Cohort I	Cohort II	Cohorts >II	Cohort I	Cohort I	Cohort I
May 29–30	**6.2 (4.3)**	**7.1 (4.5)**	**3.0 (0.7)**	**33.2**	**2.2**	**1.2**
	2.7 (1.2)	4.7 (0.7)	2.3 (1.4)	—	0.3	—
June 11–13	**3.9 (4.8)**	**7.2 (4.9)**	**2.7 (1.9)**	**64.8**	**0**	**0**
	4.5 (5.2)	10.2 (5.9)	2.5 (0.3)	—	0	—
25–27	— —	— —	— —	**67.2**	**10.6**	**0**
	— —	— —	— —	—	3.3	—
July 9–13	**5.0 (1.5)**	**9.4 (7.7)**	**1.5 (0.2)**	**26.5**	**0.3**	**0**
	1.0 (0.7)	1.8 (1.0)	1.9 (0.9)	—	0	—
23–25	**2.2 (0.7)**	**5.2 (1.0)**	**1.0 (0.8)**	**16.0**	**2.8**	**0**
	1.9 (1.5)	1.8 (1.6)	0.5 (0.2)	—	0.7	—
August 6–8	— —	— —	— —	**7.9**	**3.8**	**0**
	— —	— —	— —	—	0	—
13–14	**9.1** —	**12.2** —	**2.7** —	—	—	
	1.7 (0.2)	1.6 (1.2)	0.4 (0.0)	—	—	—
26–28	— —	— —	— —	**15.8**	**0.3**	**0.2**
	— —	— —	— —	—	0.3	—
September 2–3	**0.3 (0.4)**	**0.8 (0.7)**	**0.7 (1.0)**	—	**4.6 (3.0)**	—
	0.4 (0.1)	0.6 (0.1)	0.5 (0.4)	—	0.1 (0.2)	—
11–12	— —	— —	— —	**26.0**	**13.4**	**0**
	— —	— —	— —	—	0.3	—
24–25	**2.6 (3.5)**	**3.4 (3.3)**	**0.3 (0.2)**	**25.0**	**3.6**	**0.5**
	0.7 (1.0)	1.2 (1.1)	0.4 (0.3)	—	—	—
October 7	— —	— —	— —	**8.1**	**15.8**	**0.2**
	— —	— —	— —	—	1.5	—
13	**8.0 (7.5)**	**33.2 (32.4)**	**6.7 (6.3)**	—	—	—
	— —	— —	— —	—	—	—
20	— —	— —	— —	**11.0**	**19.0**	—
	— —	— —	— —	—	—	—
Mean:	**4.7 (3.0)**	**9.8 (10.1)**	**2.3 (2.0)**	**27.4 (20.8)**	****6.4 (6.6)**	**0.2 (0.4)**
	1.8 (1.4)	3.1 (3.3)	1.2 (1.0)	— —	0.6 (1.1)	— —

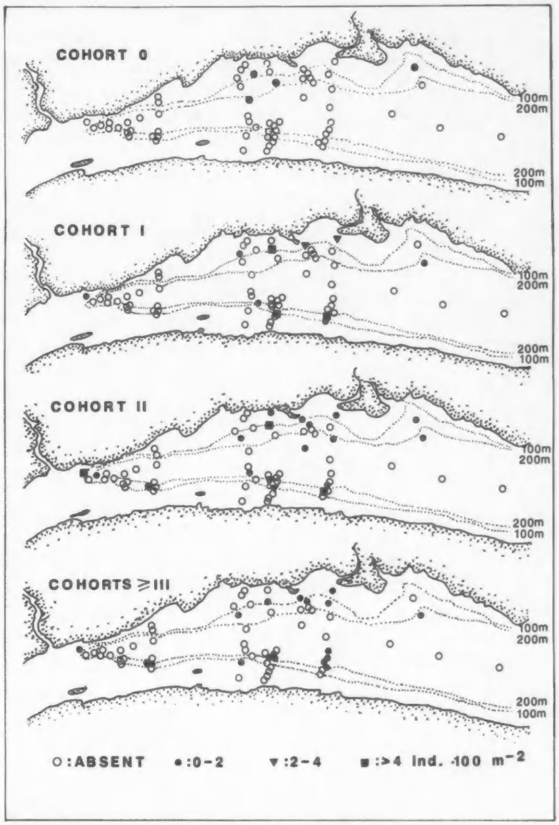

FIG. 6. Geographic distribution of cohorts 0, I, II, and >II of *Pandalus borealis* in the Lower St. Lawrence Estuary. Depth contours of 100 m and 200 m.

trend was observed. The most abundant cohort was cohort II which averaged 9.8 ind. per 100 m^2 (Table 4).

The young shrimps were often present in less abundance on the bottom at night, especially in Baie des Chaleurs (Table 4, Fig. 8), probably due to nocturnal excursions into the water column above the suprabenthic layer sampled. In the Estuary this day/night difference was masked by a high temporal variability and was therefore non-significant, except in July (Table 4).

Discussion

Length of the First Cohort

The results clearly showed that the carapace length of northern shrimp cohort I in the Gulf of St. Lawrence in summer was not larger than 10 mm, but ranged from five to 10 mm. Similarily small cohorts I have been observed in southern Labrador and Newfoundland (Parsons et al. 1986) and the Barents Sea (Teigsmark 1983). Carapace length of cohort I in Greenland fjords, computed from Horsted and Smidt's (1956) data, is also similar to that found here, if their lateral carapace length is converted to oblique carapace length (LCL ~ 1.15 OCL, from Ivanov 1964). The carapace lengths of cohort I reported by Rasmussen (1953) for the slowly growing populations of Jan Mayen and Spitsbergen are substantially higher than those reported here. The lengths attributed to cohort I for other high-latitude shrimp grounds such as those of the Bering Sea (Ivanov 1964, 1969) and the Arnarfjordur of Iceland (Skuladottir 1979) are similar to the length of our cohort II. Ivanov (1969) noted that his ages could be off by 1 yr if small individuals of the first cohort had been missed.

Previous studies in the Gulf of St. Lawrence (Simard et al. 1975) have missed what we now know to be cohort I. Present data are consistent with a pre-recruit period composed of three cohorts, the first two consisting of juveniles (0 and I) and the third (II) of young males which are mature at the end of their third year.

Slow Growth of Pre-Recruits in the Gulf of St. Lawrence

It is probable that the low-temperature waters in the Gulf of St. Lawrence were responsible for the similarity of our growth rates with those of slowly growing high-latitude populations. Because of their bathymetric distribution, cohorts I and II lived at the low temperatures (0.5–2°C) directly below the intermediate cold water mass of the Gulf of St. Lawrence. Similar low-temperature conditions were found in the high-latitude northern shrimp grounds mentioned above. This stresses the importance of temperature in controlling growth and maturation of northern shrimp populations, as suggested the pioneer works of Ras-

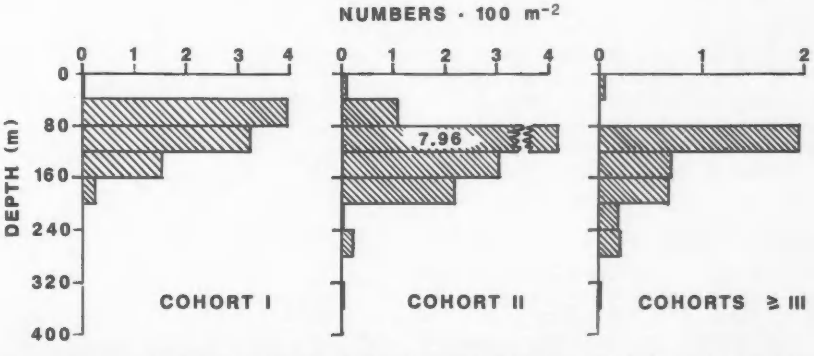

FIG. 7. Bathymetric distribution of cohorts I, II and >II of *Pandalus borealis* in the Lower St. Lawrence Estuary. Numbers of shrimps caught divided by the total surface sampled in each depth class. No. of samples = 100.

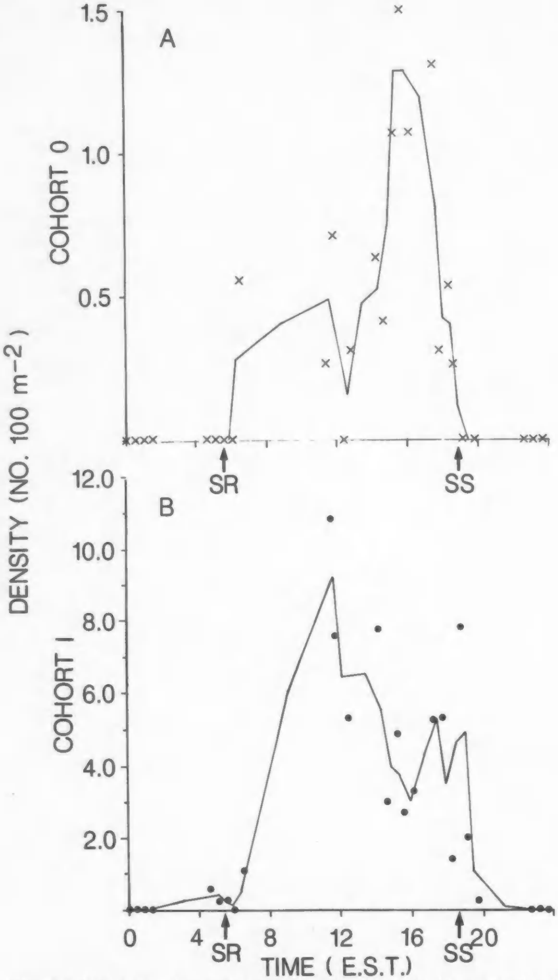

FIG. 8. Diel fluctuations of abundance of cohorts 0 (A) and I (B) of *Pandalus borealis* on the bottom at station 2 in Baie des Chaleurs on 2–3 September 1969. Moving average on 2 points. SR: sun rise, SS: sun set.

mussen (1953), Horsted and Smidt (1956), and Allen (1959). In environments where temperature is not stable over the life history of the shrimps, we can expect changes in growth rates and maturation according to temperature fluctuations on the shrimp grounds. Changes in temperature conditions experienced can also result from migrations, such as the ontogenic migration of northern shrimp to deep grounds (see Shumway et al. 1985). The first steps of this migration were evidenced here by the increase of the median depth of the cohorts with age, which also corresponded to an increase of water temperature. This ontogenic depth migration gradually moved the shrimps to the warmer (2–6°C) deep water of the Gulf of St. Lawrence; thus they did not grow at the same temperature throughout their life, but at constantly increasing temperatures. Simard and Savard (1990) have shown that this ontogenic migration along an increasing temperature gradient significantly enhances the growth rate of the older individuals compared to animals of similar age which remain in a colder water environment.

Besides temperature, season also affects growth. The observed summer peak in growth and maturation of instars indicates a close connection of shrimp growth with secondary production. Models of shrimp growth rate may therefore benefit by including a seasonal component as suggested by Boutiller et al. (1989).

Postlarval Settlement

Benthic settlement of the larvae in the Gulf of St. Lawrence appeared to occur in August–September, at larval age 4–5 mo. At the low temperatures (2–4°C, Fig. 5) of the water mass inhabited by the larvae in the Gulf (20–40 m, Ouellet et al. 1990), metamorphosis would occur at an age of 115–125 d (Shumway et al. 1985). This fits in well with the range of ages estimated from the beginning of August (110 d, early-hatched larvae) to the end of September (140 d, late-hatched larvae), given that hatching occurs from mid-April to mid-May (Ouellet et al. 1990).

Cohort 0 was much rarer than cohorts I and II, and essentially present only in August and September in Baie des Chaleurs. The transient presence of postlarvae at the 120 m deep stations 2 and 3 appears therefore exceptional, and not representative of their actual distribution and density. Extrapolating from the bathymetric distribution gradient of cohorts, cohort 0 should occur more commonly in shallow depths less than 100 m.

Spatial and Temporal Fluctuations of Density

The distribution of pre-recruit cohorts I and II appeared to be centered around the 100 m deep grounds, located on the margin of the Laurentian Channel in the two areas sampled. Their summer average density at similar depths in the two areas differed, however, by a factor of five or more. Whether this was due to temporal or spatial fluctuations cannot be determined from the present data. Semimonthly fluctuations of densities at a given station were high, up to one order of magnitude, and some of the fluctuations seemed to be related to horizontal transport. Vertical migrations into the water column during the night, implied from lower catches at night near the bottom, in combination with the circulation, may significantly contribute to horizontal displacement of shrimps.

Acknowledgments

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References

ALLEN, J. A. 1959. On the biology of *Pandalus borealis* Krøyer, with reference to a population off the Northumberland coast. J. Mar. Biol. Assoc. UK. 38: 189–220.

BERKELEY, A. A. 1930. The post-embryonic development of the common pandalids of British Columbia. Contrib. Can. Biol. Fish. N.S. 6: 79–163.

BOUTILLER, J., D. A. FOURNIER, AND J. R. SIBERT. 1989. An application of the MULTIFAN method for estimating growth parameters and age composition from multiple length frequency samples to the pink shrimp (*Pan-*

dalus jordani). Working group on shrimp ageing, NAFO SCR Doc 89/91, 22 p.

Brunel, P., M. Besner, D. Messier, L. Poirier, D. Granger, et M. Weinstein. 1978. Le traîneau suprabenthique MACER-GIROQ: appareil amélioré pour l'échantillonnage quantitatif étagé de la petite faune nageuse au voisinage du fond. Int. Rev. Gesamten Hydrobiol. 63: 815–829.

Horsted, S. A., and E. Smidt. 1956. The deep sea prawn (*Pandalus borealis* Kr.) in Greenland waters. Medd. Dan. Fisk. Havunders. 1(11): 1–118.

Ivanov, B. G. 1964. Biology and distribution of shrimps during winter in the Gulf of Alaska and the Bering Sea. Trudy VNIRO 53: 185–198. (Transl. *In* P. A. Moiseev [ed.] Soviet Fish. Invest. in the North-east Pacific, Part III, p. 176–190. Israel Progr. Sci. Transl., Jerusalem, 1968.)

1969. Biology of the northern shrimp (*Pandalus borealis* Kr.) in the Bering Sea and the Gulf of Alaska. Trudy VNIRO 65: 392–416. (Fish. Res. Board Can. Transl. Ser. 1576, 1970)

Labonté, S., and J. Fréchette. 1978. Étude de la sélectivité du chalut commercial à crevettes "Yankee 41" pour la population de *Pandalus borealis* du nord-ouest du golfe du Saint-Laurent. Travaux sur les Pêcheries du Québec 46: 19 p.

Loring, D. H., and D. J. G. Nota. 1973. Morphology and sediments of the Gulf of St. Lawrence. Bull. Fish. Res. Board Can. 182: 1–147.

Macdonald, P. D. M., and T. J. Pitcher. 1979. Age-groups from size–frequency data: a versatile and efficient method of analyzing distribution mixtures. J. Fish. Res. Board Can. 36: 987–1001.

Ouellet, P., D. Lefaivre, and V. Koutitonsky. 1990. Distribution of shrimp (*Pandalus borealis*) larvae and hydrographic patterns in the northern Gulf of St. Lawrence. Can. J. Fish. Aquat. Sci. (In press)

Parsons, D. G., G. R. Lilly, and G. J. Chaput. 1986. Age and growth of northern shrimp *Pandalus borealis* off northeastern Newfoundland and southern Labrador. Trans. Am. Fish. Soc. 115: 872–881.

Rasmussen, B. 1953. On the geographical variation in growth and sexual development of the deep sea prawn (*Pandalus borealis* Kr.). Fiskeridir. Skr. Ser. Havunders. 10(3): 1–160.

Robert, G. 1974. The sublittoral mollusca of the St. Lawrence Estuary, east coast of Canada. PhD. thesis, Inst. Oceanogr., Dalhousie University, N.S. 178 p.

Poirier, L. 1971. Biologie et écologie des Mysidacées circalittorales de l'entrée de la baie des Chaleurs (golfe du Saint-Laurent). Thèse MSc., Département sciences biologiques, Université de Montréal, Qué. 112 p.

Shumway, S. E., H. C. Perkins, D. F. Schick, and A. P. Stickney. 1985. Synopsis of biological data on the pink shrimp, *Pandalus borealis* Krøyer, 1838. U.S. Dept. of Commerce, NOAA Tech. Rep. NMFS 30: 1–57.

Simard, Y., J. Fréchette, et A. Dubois. 1975. Croissance de *Pandalus borealis* (Krøyer) dans le nord-ouest du golfe du Saint-Laurent. Min. Industr. Comm. Québec, Dir. Pêches marit., Cah. Inf. 63: 1–46.

Simard, Y., and L. Savard. 1990. Variability, spatial patterns and scales of similarity in size-frequency distributions of the northern shrimp (*Pandalus borealis*) and its migrations in the Gulf of St. Lawrence. Can. J. Fish. Aquat. Sci., 47: 794–804.

Skuladottir, U. 1979. Comparing several methods of assessing the maximum sustainable yield of *Pandalus borealis* in Arnarfjordur. Rapp. P.-V. Réun. Cons. Int. Explor. Mer 175: 240–252.

Teigsmark, G. 1983. Populations of the deep-sea shrimp (*Pandalus borealis* Krøyer) in the Barents Sea. Fiskeridir. Skr. Ser. Havunders. 17: 377–430.

Plasma Antifreeze Levels Reflect the Migratory Behaviour of Atlantic Herring (*Clupea harengus harengus*) in the Southern Gulf of St. Lawrence

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Chadwick, E. M. P., D. K. Cairns, H. M. Dupuis, K. V. Ewart, M. H. Kao, and G. L. Fletcher. 1990. Plasma antifreeze levels reflect the migratory behaviour of Atlantic herring (*Clupea harengus harengus*) in the southern Gulf of St. Lawrence. Can. J. Fish. Aquat. Sci. 47: 1534–1536.

This study compared freeze protection conferred by blood antifreeze proteins to juvenile and adult Atlantic herring (*Clupea harengus harengus*) in the southern Gulf of St. Lawrence in December 1988. Juvenile herring had significantly lower plasma freezing points and significantly higher antifreeze activity than adults. Differences in antifreeze activity and in late autumn distribution suggest that juvenile herring are more capable of wintering in the Gulf of St. Lawrence than adults. This conclusion is consistent with our observation that during December, juveniles were present in shallow waters of Chaleur Bay, which is ice-covered during winter, while adults were found only in the deeper waters of Sydney Bight, which do not freeze during winter.

La présente étude compare la protection contre le gel attribuée aux protéines sériques anti-gel chez les juvéniles et les adultes du hareng de l'Atlantique (*Clupea harengus*) dans le secteur sud du golfe du Saint-Laurent. En décembre 1988, les juvéniles montraient un point de congélation du plasma nettement inférieur et une activité anti-gel nettement supérieure aux adultes. Les différences entre l'activité anti-gel et la répartition à la fin de l'automne portent à croire que le hareng juvénile est mieux adapté à passer l'hiver dans le golfe du Saint-Laurent que le hareng adulte. Cette conclusion s'accorde avec l'observation qu'en décembre, les juvéniles étaient présents dans les eaux peu profondes de la baie des Chaleurs, recouverte de glace en hiver, tandis que les adultes n'étaient présents que dans les eaux profondes du Sydney Bight qui ne gèlent pas en hiver.

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Almost half of the projected catch of Atlantic herring (*Clupea harengus harengus*) in the southern Gulf of St. Lawrence comprises young fish newly recruited to the fishery (Chadwick et al. 1989). Consequently, knowledge of the abundance of juvenile herring is critical for stock assessment. Based on tagging studies (Winters and Beckett 1978; and W. Stobo, Department of Fisheries and Oceans, P.O. Box 1006, Dartmouth, N.S., unpubl. data), it is assumed that adult herring migrate from the southern Gulf to overwinter in deeper waters of the Laurentian Channel, Sydney Bight and possibly southwest Newfoundland. Juvenile herring are resident in the Gulf in June–November and there are scattered records in December–March (H. M. C. Dupuis et al. unpubl. data; Cairns 1989). However, it is not known if most juveniles leave the southern Gulf to winter in less frigid waters.

Blood antifreeze proteins are found in a number of fish species, including Atlantic herring, which are associated with ice-laden, subzero water temperatures (Davies et al. 1988; Ewart and Fletcher 1990). These proteins protect such fish from freezing during winter (Fletcher et al. 1986). Recently two of us found that the levels of antifreeze proteins were higher in juvenile Atlantic cod (*Gadus morhua*) than adults, suggesting that juveniles could overwinter in cold water near the cost of Newfoundland, but adults must migrate offshore (Kao and Fletcher 1988).

The objective of this study was to compare freeze protection conferred by blood antifreeze proteins of juvenile and adult herring just prior to winter in the southern Gulf of St. Lawrence. The null hypothesis was that freeze protection was equal in juveniles and adults and the groups had similar overwintering migratory behaviour.

Methods

Atlantic herring were collected from the research vessel *E.E. Prince* during an acoustic survey in the southern Gulf, December 1988. This survey was designed to estimate the distribution and biomass of juvenile and adult herring. In addition, surface-to-bottom sea temperatures were determined using expendable bathythermograph casts at a grid of sites in Chaleur Bay and Sidney Bight. Details of the survey, including maps of transects, length–frequencies of samples collected and temperature profiles are summarized in Cairns et al. (1989). Esti-

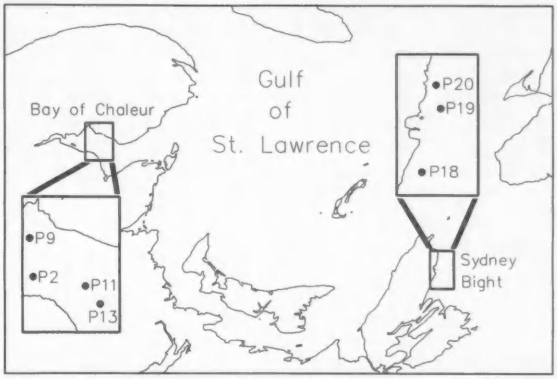

FIG. 1. Southern Gulf of St. Lawrence, showing locations of sets made to capture juvenile and adult herring in the Bay of Chaleur and Sydney Bight. December 1988.

mates of biomass and distribution of juvenile herring will be published elsewhere.

An Engel trawl was used to determine species composition and size structure of insonified targets and to collect herring for blood samples. The sets were 30 min at 3.5 knots and in Chaleur Bay were made within several meters of the bottom. In Sydney Bight, sets were made approximately midway between surface and bottom. Location of the sets is given in Fig. 1; the depths of these sites in Table 1. Sampling was proportional to the total acoustic backscatter and therefore was probably representative of the area surveyed. Juvenile herring (fish < 200 mm) were widely distributed in the upper part of Chaleur Bay and blood samples were collected from samples of live herring taken in four sets (4–7 December, 1988). Adult herring were found only in the Sydney Bight area (Fig. 1) and blood samples were collected from random samples of herring taken in three sets (12–13 December, 1988).

Blood samples were taken from a caudal blood vessel using two or three heparinized micro-pipettes. The red cells were removed from the plasma by centrifugation (300 *g*). The plasma was frozen and transported to the Ocean Sciences Centre for determination of freezing and melting points. Total length of each fish in the set was measured to the nearest millimetre. Age and spawning group were determined by examination of otoliths from random-stratified subsamples of other herring taken in the sets.

Plasma freezing and melting points were determined using a Clifton nanolitre freezing point osmometer (Clifton Technical Physics, Hartford, NY) Kao et al. 1986). The difference between the freezing and melting points, termed thermal hysteresis, is a direct measure of antifreeze activity and an indication of antifreeze protein levels. Student's *t*-test was used to compare fish size and plasma freezing and melting points between juveniles and adults.

Results and Discussion

Plasma freezing points were significantly lower and antifreeze activity significantly higher in juvenile herring than in adults (Fig. 2). Mean thermal hysteresis of juveniles was nearly three times higher than that of adults, which indicated that juveniles had significantly more antifreeze.

There were slight differences in water temperature between sites. Juvenile herring in Chaleur Bay were collected at 2–3°C, while adult herring in Sydney Bight were collected at 3–4°C. Temperatures below 1°C have been found to stimulate formation of antifreeze in adult cod (Fletcher et al. 1987). Although we do not know what stimulates antifreeze production in herring, it is unlikely that the marginally cooler waters in Chaleur Bay would have triggered greater antifreeze activity.

Within areas, variation in blood antifreeze levels did not significantly covary with fish size (Table 1). Consequently, size alone could not explain differences in antifreeze activity between the two areas.

The results also suggested that antifreeze activity was related to stock structure. Almost all juveniles sampled in Chaleur Bay belonged to the spring-spawning stock, while most adults from Sydney Bight were fall spawners (Table 1). The large concentration of juveniles from the spring-spawning stock was unexpected because two-thirds of the herring biomass in the southern Gulf are fall spawners (Chadwick et al. 1989). Thus, it would appear that more work needs to be done towards locating and sampling the overwintering aggregations of spring-spawning adults and fall-spawning juveniles.

All evidence indicates that juveniles were overwintering in Chaleur Bay. First, in a similar survey during November 1988 both juveniles and adults were present in the bay (Cairns et al. 1989); during an interval of one month adults had migrated out of the bay, but juveniles remained. The adults in the November survey comprised equal components of spring and fall spawners (Chadwick et al. 1989). Second, it appeared that juveniles were restricted to a lens of 2–4°C water surrounded by much cooler

TABLE 1. Comparison of plasma freezing characteristics, length and spawning group of juvenile and adult Atlantic herring. Juvenile herring were less than 200 mm in length. Water depth and temperature for each set are also shown.

Age	Site	Water depth (m)	Water temperature (°C)	Thermal hysteresis (°C)			Total length (m)			Spring spawners (%)
				n	$\bar{x}$	SE	*n*	$\bar{x}$	SE	
Juvenile	P2	18	1.5	22	0.081	0.015	29	119	2.3	100
	P9	25	2.0	16	0.166	0.019	83	124	1.8	94
	P11	34	2.5	18	0.172	0.021	18	127	1.3	100
	P13	51	2.5	9	0.083	0.027	18	151	2.9	94
	Combined			65	0.128	0.011				
Adult	P18	110	3.5	16	0.026	0.009	160	302	2.4	32
	P19	100	3.5	16	0.088	0.017	87	302	2.8	22
	P20	80	2.5	13	0.028	0.008	355	268	2.8	46
	Combined			45	0.0486	0.008				

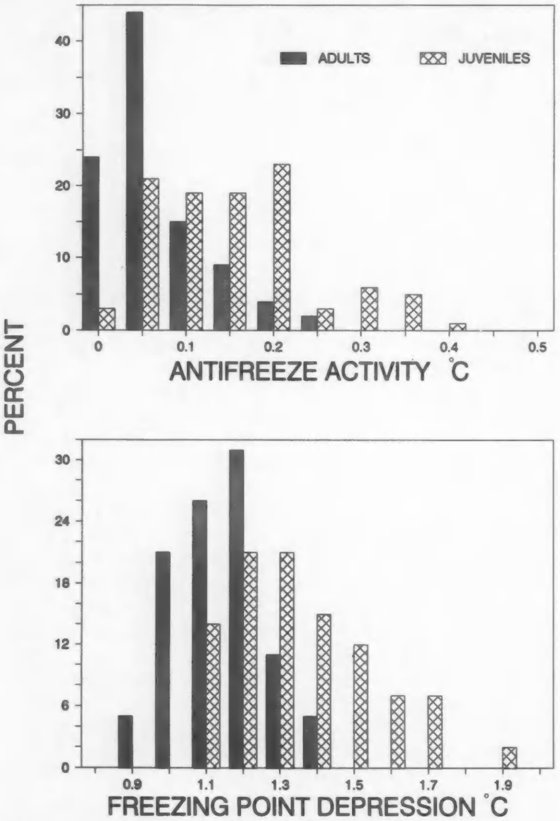

FIG. 2. Frequency distributions of plasma antifreeze activity, and plasma freezing point depression in juvenile (n = 65) and adult (n = 45) Atlantic herring.

water in the outside part of the bay. Because temperature in Chaleur Bay was cooling quickly at the time of the December survey, there appeared to be no temperature window to provide access out of the bay before freeze up. This preliminary study does not indicate the full extent to which herring can become freeze resistant. Nevertheless, it does establish that juveniles are more prepared to overwinter under freezing conditions at an earlier date than adults.

Antifreeze protection has also been found in adult Pacific herring (*Clupea harengus pallasi*) in frigid Alaskan and Japanese waters (Raymond 1989). Some populations of northwest Atlantic herring may also generate antifreeze proteins while adults, as herring caught in the under-ice fishery of the northeast coast of Newfoundland presumably require such protection. Brawn (1960) found that herring from southwestern New Brunswick were able to survive brief exposures to − 1.1°C seawater. However, this does not indicate that such fish contained antifreeze proteins (Davies et al. 1988).

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References

BRAWN, V. M. 1960. Temperature tolerance of acclimated herring (*Clupea harengus* L.). J. Fish. Res. Board Can. 17: 721–723.

CAIRNS, D. K. 1989. Gear types, seasonal distribution of effort, and bycatch of the smelt fishery in the southern Gulf of St. Lawrence. Can. Tech. Rep. Fish. Aquat. Sci. 1668: 20 p.

CAIRNS, D. K., J. A. WRIGHT, AND E. M. P. CHADWICK. 1989. Distribution and acoustic backscatter of herring in NAFO Divisions 4T and 4Vn, November–December 1988. Can. Atl. Fish. Sci. Adv. Comm. Res. Doc. 89/42: 51 p.

CHADWICK, E. M. P., D. K. CAIRNS, AND H. M. DUPUIS. 1989. Assessment of Atlantic herring in NAFO Division 4T, 1988. Can. Atl. Fish. Sci. Adv. Comm. Res. Doc. 89/63: 60 p.

DAVIES, P. L., C. L. HEW, AND G. L. FLETCHER. 1988. Fish antifreeze proteins: physiology and evolutionary biology. Can. J. Zool. 66: 2611–2617.

EWART, K. V., AND G. L. FLETCHER. 1990. Isolation and characterization of antifreeze proteins from smelt (*Osmerus mordax*) and Atlantic herring (*Clupea harengus harengus*). Can. J. Zool. (In press)

FLETCHER, G. L., M. H. KAO, AND R. M. FOURNEY. 1986. Antifreeze peptides confer freezing resistance to fish. Can. J. Zool. 64: 1897–1901.

FLETCHER, G. L., M. J. KING, AND M. H. KAO. 1987. Low temperature regulation of antifreeze glycopeptide levels in Atlantic cod (*Gadus morhua*). Can. J. Zool. 65: 227–233.

KAO, M. H., AND G. L. FLETCHER. 1988. Juvenile Atlantic cod (*Gadus morhua*) can be more freeze-resistant than adults. Can. J. Fish. Aquat. Sci. 45: 902–905.

KAO, M. H., G. L. FLETCHER, N. C. WANG, AND C. L. HEW. 1986. The relationship between molecular weight and antifreeze polypeptide activity in marine fish. Can. J. Zool. 64: 578–582.

RAYMOND, J. A. 1989. Freezing resistance in some northern populations of Pacific herring, *Clupea harengus pallasi*. Can. J. Fish. Aquat. Sci. 46: 2104–2107.

WINTERS, G. H., AND J. S. BECKETT. 1978. Migrations, biomass and stock interrelationships of Southwest Newfoundland – Southern Gulf herring from mark-recapture experiments. Int. Comm. Northwest Atl. Fish. Res. Bull. 13: 67–79.

Controls on Dissolved Organic Carbon Concentrations in Streams, Southern Québec

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Eckhardt, B. W., and T. R. Moore, 1990. Controls on dissolved organic carbon concentrations in streams, southern Québec. Can. J. Fish. Aquat. Sci. 47: 1537-1544.

Dissolved organic carbon (DOC) concentrations were measured in streams draining 42 small (0.6–37.2 km^2) catchments located in the Appalachian Uplands and St. Lawrence Lowlands. Weekly sampling from mid-April to late-November in four catchments containing <1% wetland revealed DOC concentrations in streams averaging 3.5–7.2 $mg \cdot L^{-1}$ with significant ($r^2 = 0.28$–0.66, $P<0.01$) positive relationships to discharge. In four catchments containing 15–69% wetland, average DOC concentrations in streams ranged from 14.5 to 40.0 $mg \cdot L^{-1}$ and there was no significant relationship to discharge. Based on five sampling dates from May to November, DOC concentrations in streams draining the 42 catchments showed consistent relationships of varying strength ($r^2 = 0.26$–0.67, $P<0.01$) with the variable percent wetland in the catchment. Soil drainage rating, percent forest, mean catchment slope, and catchment area provided little improvement in the regression model. Separating the catchments into two regions (Uplands and Lowlands) improved the predictive power of the regression model for the upland catchments. The poor relationships between DOC and percent wetland in the lowland catchments are attributed to the dry summer during sampling and extensive modification of land use, such as the drainage of wetlands and agriculture. These results indicate that stream DOC concentrations may be predicted from easily-obtained catchment variables, such as percent wetland.

Des concentrations de carbone organique dissous (COD) ont été mesurées dans des cours d'eau drainant 42 petits bassins versants (0,6 à 37,2 km^2) situés dans les hautes terres des Appalaches et dans les basses-terres du Saint-Laurent. D'après les échantillonnages effectués de la mi-avril jusqu'à la fin de novembre dans quatre bassins versants contenant moins de 1 % de terres humides, les concentrations de COD dans les cours d'eau s'établissaient en moyenne entre 3,5 et 7,2 $mg \cdot L^{-1}$ avec un rapport positif significatif (r^2 compris entre 0,28 et 0,66 $P < 0{,}01$) avec le débit. Dans le cas des quatre bassins versants composés de 15 à 69 % de terres humides, les concentrations moyennes de COD dans les cours d'eau étaient comprises entre 14,5 et 40,0 $mg \cdot L^{-1}$ et il n'existait aucun rapport significatif avec le débit. D'après les cinq dates de prélèvements de mai à novembre, les concentrations de COD dans les cours d'eau drainant les 42 bassins versants présentaient un rapport uniforme de force variée (r^2 compris entre 0,26 et 0,67, $P < 0{,}01$) avec le pourcentage (%) variable de terres humides dans le bassin versant. Le taux de drainage des sols, le pourcentage de forests, la pente moyenne du bassin versant et le bassin versant ont apporté peu d'améliorations au modèle de régression. La division des bassins versants en deux régions (les hautes terres et les basses-terres) a amélioré l'efficacité prédictive du modèle de régression pour les bassins versants des hautes terres. Le faible rapport entre le COD et le pourcentage (%) de terres humides dans les bassins versants des basses-terres est attribué à la sécheresse de l'été pendant l'échantillonnage et à une modification poussée de l'utilisation des terres, comme le drainage des terres humides et les pratiques agricoles. Ces résultats montrent que les concentrations de COD dans les cours d'eau peuvent être prévues à partir de variables facilement obtenues sur le bassin versant, comme le pourcentage de terres humides.

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Dissolved organic carbon (DOC) is an imprtant characteristic of water quality, through its influence on acidity, the mobility and transport of metals, light penetration, and the productivity of aquatic organisms (e.g. Driscoll et al. 1989; Suffet and MacCarthy 1989; Thurman 1985; Urban et al. 1989). Within catchments, the main sources of DOC are precipitation and the leaching of plant material and organic soil horizons (e.g. Moore 1989). Dissolved organic carbon is often adsorbed by subsoil horizons rich in iron and aluminum (e.g. McDowell and Likens 1988; Moore 1989) and DOC can be released into streams through the decomposition of organic matter followed by DOC absorption on channel sediments (McDowell 1985) or biotic uptake (e.g. Dahm 1980). The controls on DOC concentrations in streams include the sources and sinks within the catchment and the hydrologic pathways whereby the DOC is transported to the stream.

Numerous studies of DOC concentrations in streams have been made, showing concentrations ranging from <2 to >50 $mg\ DOC \cdot L^{-1}$. However, many studies have dealt with only one or two catchments, thus limiting the ability to generalize. Furthermore, few studies have examined which attributes of the catchment are related to the concentration of DOC in streams and, in turn, whether easily-determined catchment attributes can be used for prediction of these concentrations.

Moeller et al. (1979), for example, showed that DOC export, rather than DOC concentration, could be related to link magnitude and catchment area and other characteristics in a series of watersheds in the United States. Mulholland and Kuenzler

(1979) related DOC export to two main groups of catchment, upland and swampy, and Urban et al. (1989) have extended this relationship. The focus on DOC export, by including hydrologic variability, does not allow an examination of the relationship between DOC concentration and catchment characteristics. Within lakes, Ensgtrom (1987) has been able to relate lake water colour (a surrogate for DOC concentration) in Labrador to characteristics of the catchment such as vegetation and hydrology. Rasmussen et al. (1989) have recently shown that lake water colour can be predicted from simple morphometric characteristics of the lake and its catchment.

In this paper, we examine variations in the DOC concentration of streams draining eight small catchments in southern Quebec, four of which contain substantial areas of wetland and four of which contain predominantly well-drained soils. We relate the variations in DOC within the streams to changes in discharge and season and examine the influence of catchment characteristics on mean DOC concentrations. By extending the analysis to 34 other catchments covering a wider range of environments in southern Québec, we test the hypothesis that DOC concentrations in streams can be predicted from simple catchment characteristics and examine inter-regional differences in these relationships.

Materials and Methods

Forty-two catchments were selected in southern Québec, based on criteria of size, soil type, and land use and accessibility (Table 1). Twenty-five of the catchments were located in the Appalachian Uplands region with rolling topography, soils developed on till, and fluvioglacial materials derived from metamorphic and igneous rocks. Land use was a mixture of forests and extensive agriculture, such as pasture. The other 17 catchments were located in the St. Lawrence Lowlands, where the topography is generally flat, the soils are developed on sediments laid down by the Champlain Sea, forest cover is less extensive, and agricultural crops such as corn are grown on the most fertile, well-drained soils. In southern Québec, mean annual precipitation is about 1 000 mm, of which 500–600 mm runs off, and mean daily temperatures in July reach about 18°C (Energy, Mines and Resources Canada 1974).

Eight of the catchments in the Rivière Noire — Rivière Yamaska catchments were selected for more detailed study: four contained dominantly well-drained soils (catchments 1–4) and the other four possessed significant proportions of wetland (catchments 5–8, Table 1). The streams draining these catchments were sampled at approximately weekly intervals from April to November, 1988. Discharge at the time of sampling was measured with a current meter or the float-velocity method and measured channel dimensions.

Each of the 42 catchments was characterised as follows:
Area: from topographic maps;
Wetland percentage: from the proportion of the catchment covered by bog, fen, swamp, and beaver pond, derived from topographic and soil maps and aerial photographs;
Drainage: based on the county soil survey reports (Baril and Mailloux 1950; Cann et al. 1947; Mailloux and Godbout 1954), an average drainage rating for the catchment was obtained by calculating the proportion of the catchment covered by soils with different drainage classes. This criterion does not include artificial drainage, e.g. for farming. Each drainage class was weighted as follows:

Very poor	0
Poor	1
Imperfect	2
Moderate	3
Good	4
Excellent	5

Slope: the average slope in the catchment was calculated as follows —

$$\text{slope }(\text{m}\cdot\text{km}^{-1}) = \frac{\Delta\text{ altitude}}{\sqrt{(\text{area}/\pi)}}$$

where Δ altitude = average difference in elevation from watershed boundary to the stream channel (m), at sampling point
area = area of catchment (km^2)

Forest: aerial photographs and topographic maps were used to determine the percentage of the catchment covered by forest.

To provide data to test a model that predicted stream DOC concentration from simple catchment characteristics, the complete set of 42 catchments was sampled on the same 5 dates from July, 1988 to May, 1989.

Stream water samples were filtered through Whatman GF/C glass fibre filters and stored at 2–4°C. They were analysed for DOC by the dichromate digestion method (Moore 1985). Specific conductance at 25°C and pH were also determined. Two samples from each of the eight main catchments and one sample from the wider group were analysed for cations and anions to characterise their geochemistry.

Results

Controls on Stream DOC Concentrations in the Eight Catchments

In the non-wetland catchments, DOC concentrations ranged from 1.7 to 13.8 $\text{mg}\cdot\text{L}^{-1}$, with mean values from 3.5 to 7.2 $\text{mg}\cdot\text{L}^{-1}$ (Fig. 1, Table 2). The highest mean DOC concentration was observed in catchment 3, in which the forest vegetation is mainly coniferous and the soils are commonly shallow and stony, derived from sandstone and shale materials.

In contrast, DOC concentrations in the streams draining the wetland catchments ranged from 8.5 to 73.4 $\text{mg}\cdot\text{L}^{-1}$, with mean values of 14.5–40.0 $\text{mg}\cdot\text{L}^{-1}$. Catchment 5 had the lowest mean DOC concentration, probably associated with a steeper slope and more farmland than in the other wetland catchments. Although the greatest absolute variability in DOC concentrations was observed in the wetland catchment streams, the highest relative variability occurred in the non-wetland streams. The coefficients of variation in catchments 1–4 ranged from 0.33 to 0.36, compared to 0.22–0.27 in catchments 5–8.

The streams were weakly acidic to circumneutral, with mean pH values ranging from 5.9 to 6.1 and specific conductances of 36–153 $\mu\text{S}\cdot\text{cm}^{-1}$ (Table 2). There was a significant relationship between mean DOC and mean pH and mean DOC and mean specific conductance between the 8 catchments ($r^2=0.88$ and 0.62, $P=0.001$ and 0.021, respectively). This relationship did not hold, however, for within-stream samples, with only catchment 4 showing a significant relationship ($P<0.01$) between DOC and pH and specific conductance over the sampling period.

Much of the variability in DOC concentrations within the non-wetland catchments was explained by variations in

TABLE 1. Characteristics of the 42 catchments. Catchments 1–25 are located in the Appalachian Uplands and 26–42 in the St. Lawrence Lowlands.

Catchment	Location[a]	Area (km^2)	Wetland (%)	Slope ($m \cdot km^{-1}$)	Drainage[b]	Forest (%)
1	093332 31H/8	5.2	0.0	25.5	4.0	42.9
2	096359 31H/8	3.0	0.0	24.5	2.5	20.0
3	962272 31H/8	3.5	0.5	23.2	3.0	69.7
4	128312 31H/8	7.0	0.5	30.5	3.0	75.9
5	062371 31H/8	8.5	15.0	24.0	1.0	60.0
6	065522 31H/8	7.5	23.0	10.9	1.5	84.9
7	008290 31H/8	8.7	31.1	18.9	1.5	83.5
8	024423 31H/9	9.0	68.8	8.4	1.0	92.9
9	049279 31H/8	6.5	0.0	37.6	2.5	81.4
10	107377 31H/8	2.3	0.0	45.7	2.0	11.1
11	990356 31H/8	3.1	0.0	45.4	3.0	63.9
12	838027 31H/2	3.2	0.0	30.5	2.5	60.0
13	886054 31H/2	37.2	0.7	49.5	4.5	88.0
14	885208 31H/7	13.3	0.8	29.7	1.5	63.0
15	053193 31H/8	4.1	1.0	28.0	3.0	60.0
16	795254 31H/7	5.0	2.2	14.6	3.8	60.0
17	081281 31H/8	7.0	4.5	30.5	3.0	75.9
18	830979 31H/2	19.4	5.8	18.5	2.0	80.0
19	765006 31H/2	17.0	6.6	11.0	2.0	75.0
20	024172 31H/8	1.7	8.8	45.1	3.0	20.0
21	891182 31H/7	8.7	11.7	30.8	4.0	55.0
22	933177 31H/7	4.3	17.6	44.9	2.0	87.2
23	090487 31H/9	6.0	21.0	36.1	2.5	69.9
24	965455 31H/9	0.6	44.0	7.1	1.0	94.7
25	829454 31H/10	8.8	55.9	5.5	1.0	69.9
26	443952 31H/3	5.2	0.0	2.4	1.5	7.5
27	472955 31H/3	4.6	2.0	2.5	2.5	15.0
28	887918 31H/4	25.5	10.4	12.5	3.8	55.0
29	119953 31H/3	4.4	21.4	6.6	3.5	40.0
30	433914 31H/3	1.8	24.3	5.0	1.3	39.0
31	906951 31H/4	0.6	25.0	7.0	2.6	35.0
32	053946 31H/4	5.2	25.6	6.3	0.0	49.9
33	856904 31H/4	4.9	28.5	4.4	2.7	74.9
34	144063 31H/4	8.3	29.0	1.3	1.5	49.9
35	141060 31H/4	8.7	33.0	2.7	1.3	20.0
36	404913 31H/4	11.0	38.5	1.3	1.3	65.0
37	446951 31H/3	4.0	47.0	4.2	1.0	75.0
38	320020 31H/3	20.0	51.3	1.3	1.0	40.0
39	048955 31H/4	4.0	52.8	7.4	2.8	55.0
40	052957 31H/4	31.6	56.1	2.4	0.0	55.1
41	144945 31H/4	4.2	56.4	1.8	1.0	70.0
42	018956 31H/4	6.2	79.0	1.1	0.0	55.0

[a] UTM grid reference and NTS map sheet number (1:50000).

[b] Average rating of drainage class (0–5).

discharge. There was a significant ($P<0.01$) positive relationship between DOC and discharge, with r^2 values ranging from 0.28 to 0.66 for ln DOC:ln discharge (Table 3). In contrast, discharge explained a very small proportion of the variation in DOC concentration in the wetland catchment streams ($P>0.05$, $r^2=0.00$–0.09, Table 3).

Stream DOC concentration was not obviously related to season. To evaluate the role of seasonality separate from discharge, the residuals from the ln DOC:ln discharge regression were plotted against DOY. In three of the non-wetland catchments, there was a distinct seasonal trend, with positive residuals during the spring and fall and negative residuals during the summer. Such a pattern did not exist in the fourth non-wetland catchment (3) and in the wetland catchments.

Catchment Characteristics as Predictors of DOC Concentrations

We observed a significant difference in median stream DOC concentrations between the four wetland and four non-wetland catchments (NPAR1WAY-test, $P<0.05$, SAS 1985). This difference indicates that simple catchment characteristics may be used to predict stream DOC concentrations.

Multiple and stepwise linear regression was used to model the relationship between DOC concentration and catchment variables in the 42 catchments over the five sampling dates. DOC concentration was transformed to the natural logarithm, to produce a normal distribution. Analysis of the variables for the 42 catchments (Table 1) revealed that there were significant ($P<0.01$) correlations between percent wetland and slope and drainage, but not with area and percent forest. Collinearities

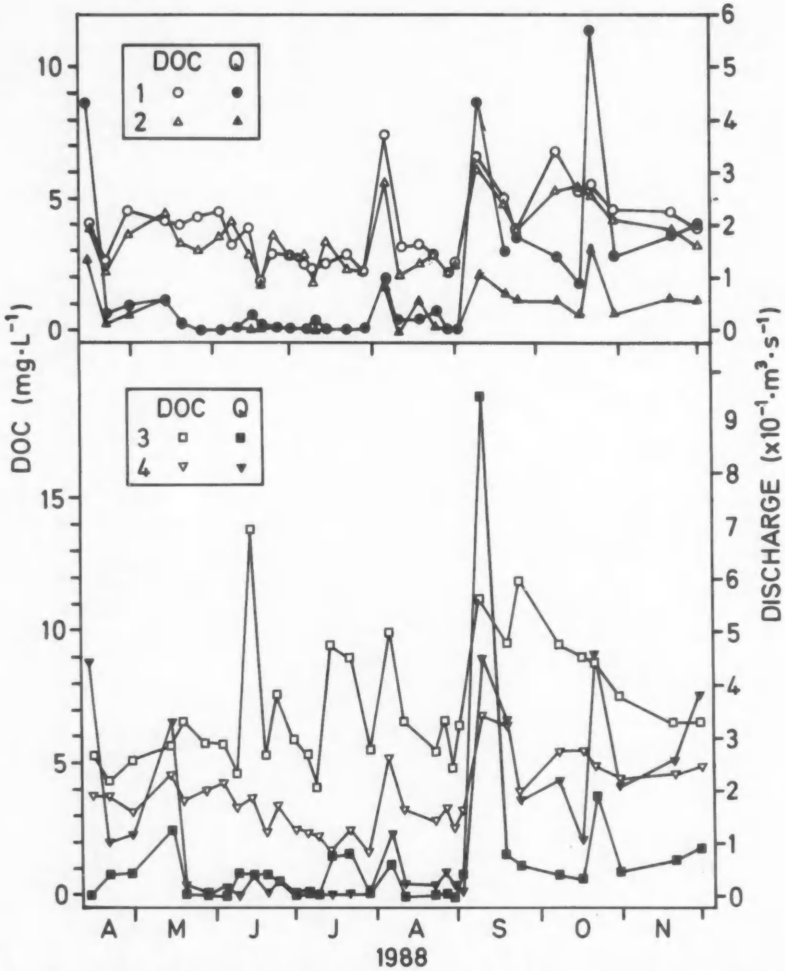

FIG. 1. Variation of stream DOC concentration and discharge (Q) from weekly sampling of the four non-wetland catchments.

TABLE 2. Mean DOC concentration, specific conductance at 25°C and pH of stream water samples from the eight catchments selected for intensive study, based on weekly sampling from April to November, 1988. Figures in parentheses indicate the standard deviation.

Catchment	DOC ($mg \cdot L^{-1}$)	Specific conductance ($\mu S \cdot cm^{-1}$)	pH
1	3.9 (1.4)	131 (40)	7.1 (0.4)
2	3.5 (1.2)	153 (36)	7.1 (0.5)
3	7.2 (2.4)	91 (21)	6.7 (0.4)
4	3.8 (1.3)	70 (19)	6.8 (0.4)
5	14.5 (3.4)	78 (15)	6.5 (0.4)
6	32.1 (7.1)	48 (4)	6.3 (0.6)
7	21.9 (6.0)	40 (9)	6.3 (0.5)
8	40.0 (9.0)	36 (13)	5.9 (0.6)

incurred in the regression of the five variables against DOC were regarded as minor, based on the condition number index (PROC REG, SAS 1985). Catchment area and percent forest showed no association ($r^2 = 0.00$) with stream DOC and were dropped from the multiple regression analysis.

A model with 1n DOC as the dependent variable was then run for the five sampling dates in each of the 42 catchments. The results showed that the percent wetland variable was able to explain the highest proportion of the DOC variance, ranging from 0.26 to 0.67 and with consistent, positive regression coefficients (0.011–0.022), significantly different from zero (Student's *t*-test, $P<0.05$). Soil drainage and slope made smaller contributions to the regression, ($r^2 = 0.12$–0.39 and 0.11–0.38, respectively) and their regression coefficients were less consistent, being generally negative.

Dividing the streams into regions representing the Appalachian Uplands and the St. Lawrence Lowlands revealed that, in general, the relationships between stream DOC and catchment variables were strongest in the Upland catchments ($r^2 = 0.37$–0.79). The Lowland catchments showed weaker DOC:catchment variable relationships ($r^2 = 0.13$–0.60). This weakness is probably associated with the more complex land

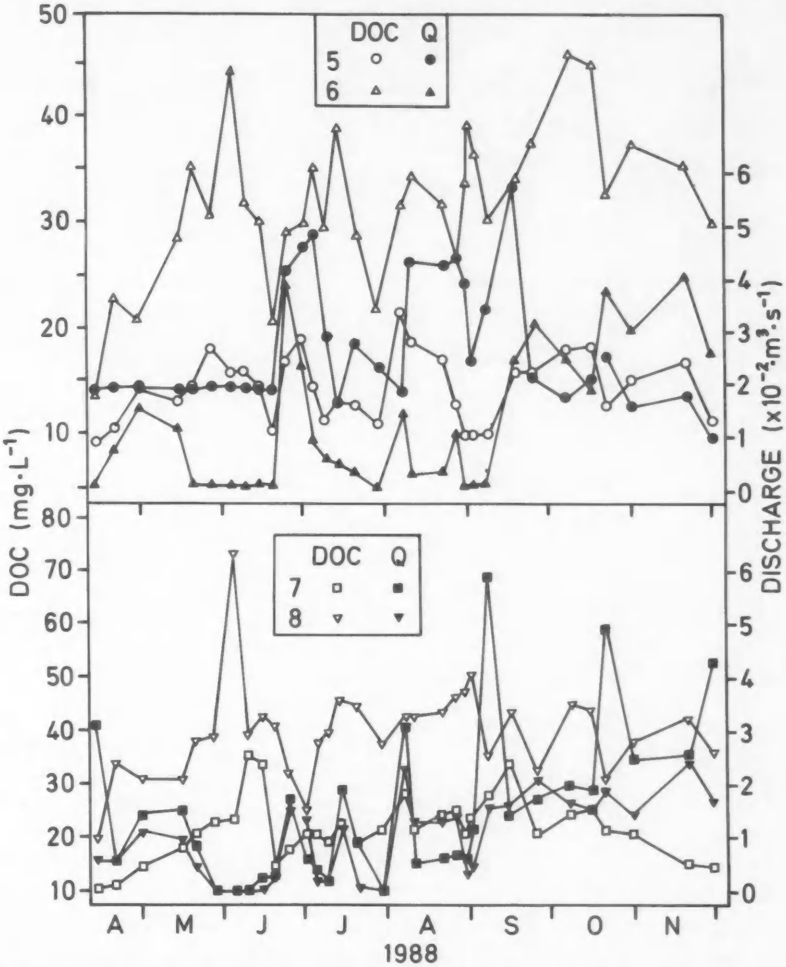

FIG. 2. Variation of stream DOC concentration and discharge (Q) from weekly sampling of the four wetland catchments.

TABLE 3. Relationships between DOC concentration (natural logarithm, mg·L^{-1}) in stream water and discharge (Q, natural logarithm, m^3·s^{-1}) in the eight catchments, based on weekly sampling from April–November, 1988 ($n=32$).

Catchment	Regression	r^2	SE_{est}	Sig. level
1	ln DOC = 1.73 + 0.11 ln Q	0.42	0.11	0.0001
2	ln DOC = 1.83 + 0.15 ln Q	0.53	0.12	0.0001
3	ln DOC = 2.33 + 0.10 ln Q	0.28	0.12	0.0036
4	ln DOC = 1.83 + 0.19 ln Q	0.66	0.08	0.0001
5	ln DOC = 2.69 + 0.15 ln Q	0.00	0.15	0.7393
6	ln DOC = 3.54 + 0.03 ln Q	0.05	0.08	0.2395
7	ln DOC = 2.90 − 0.06 ln Q	0.09	0.10	0.1078
8	ln DOC = 3.59 − 0.02 ln Q	0.01	0.09	0.5354

use and land use change and with the more diverse geology and soils which are mainly till-derived in the Uplands, but derived from outwash sands and Champlain Sea clays in the Lowlands. Stream water samples from the Lowland catchments had a higher specific conductance (mean values 167–606μS·cm^{-1}) than in the Upland catchments (36–320 μS·cm^{-1}).

As percent wetland showed the strongest relationship to stream DOC concentration and is the easiest of the catchment variables to determine from topographic and soil maps and air photographs, a regression was run for the five sampling dates (Table 4). The regressions were all significant at the $P<0.01$ level, with r^2 values ranging from 0.30 to 0.67. Mallow's C_p statistic indicated that the one variable model of DOC concentration was most statistically appropriate (SAS 1985). The range of intercept values was small (1.82–2.13, equivalent to DOC concentrations of 6.2–7.6 mg·L^{-1}), the regression coefficients ranged from 0.017 to 0.027 and were significantly different from zero (Student's t-test, $P<0.01$) and the standard errors of the estimate varied from 0.09 to 0.17. A graphical illustration of the relationships for the May sampling is given in Fig. 3. The two Lowland catchment outliers from the regression

TABLE 4. Regression between stream DOC concentration (ln DOC, mg·L^{-1}) and percent wetland (X) for the 42 catchments sampled at five dates. $\mathrm{SE_{est}}$ = standard error of the estimate; Sig. level = significance level of the regression coefficient; C = regression of combined Upland and Lowland catchments; L = regression of Lowland catchments; U = regression of Upland catchments.

Date	Equations	r^2	$\mathrm{SE_{est}}$	Sig. level	Number of catchments
Aug. 4 1988	$\ln \mathrm{DOC} = 1.82 + 0.023X$	0.30	0.17	0.0004	42 (*C*)
	$= 2.52 + 0.000X$	0.00	0.47	0.9800	17 (*L*)
	$= 1.69 + 0.041X$	0.52	0.17	0.0001	25 (*U*)
Sept. 21 1988	$\ln \mathrm{DOC} = 2.03 + 0.017X$	0.26	0.14	0.0005	42 (*C*)
	$= 1.90 + 0.022X$	0.15	0.38	0.1199	17 (*L*)
	$= 2.04 + 0.033X$	0.54	0.13	0.0001	25 (*U*)
Oct. 19 1988	$\ln \mathrm{DOC} = 1.98 + 0.021X$	0.33	0.15	0.0001	42 (*C*)
	$= 1.84 + 0.074X$	0.21	0.39	0.0700	17 (*L*)
	$= 1.98 + 0.031X$	0.57	0.13	0.0001	25 (*U*)
Nov. 30 1988	$\ln \mathrm{DOC} = 1.85 + 0.027X$	0.67	0.09	0.0001	42 (*C*)
	$= 2.02 + 0.022X$	0.59	0.21	0.0003	17 (*L*)
	$= 2.06 + 0.022X$	0.31	0.16	0.0041	25 (*U*)
May 2 1989	$\ln \mathrm{DOC} = 1.96 + 0.024X$	0.57	0.10	0.0001	42 (*C*)
	$= 2.15 + 0.023X$	0.45	0.25	0.0031	17 (*L*)
	$= 1.90 + 0.022X$	0.62	0.08	0.0001	25 (*U*)

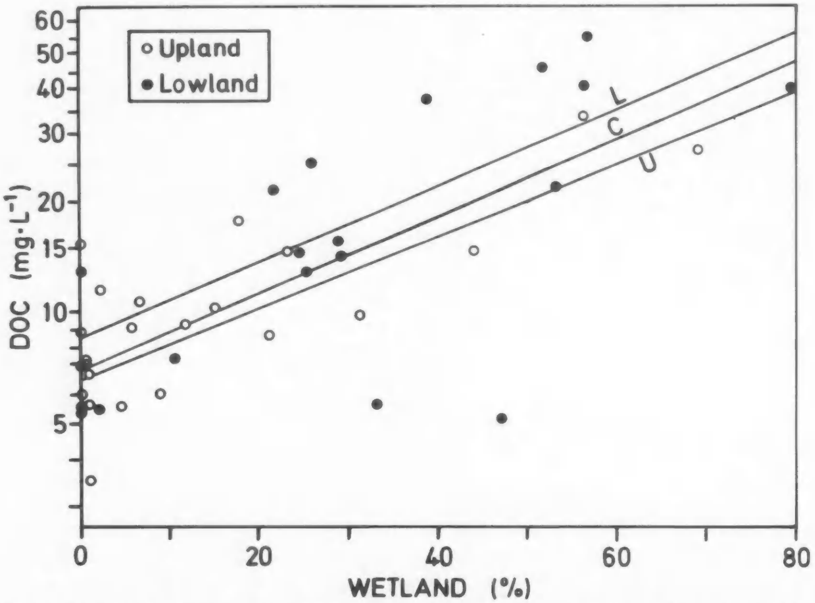

FIG. 3. Relationship between stream DOC concentration and percentage of the catchment covered by wetland in the 42 catchments, sampled on May 2, 1989. Lines represent regression equations listed in Table 4, with *L* being the Lowland catchments, *U* the Upland catchments and *C* the combined regression.

have been extensively drained for agriculture, probably changing sources and transport of DOC to the stream.

Discussion

The sources, sinks, and pathways of DOC in terrestrial systems are now reasonably well identified, though quantification of these fluxes remains to be established for many ecosystems (cf. McDowell and Likens 1988; Meyer and Tate 1983; Moore 1989; Moore and Jackson 1989). DOC enters the catchment through precipitation and the leaching and decomposition of vegetation and soil organic horizons. DOC-rich water percolating into the subsoil can be adsorbed by the soil horizons or pass through with little change in concentration (e.g. Cronan and Aiken 1985; McDowell and Wood 1984). In catchments where water flow is through the organic soil horizons, through shallow soils, or through overland flow, much of the DOC produced by the vegetation and soil can reach the stream channel. Concentrations of DOC in streams represent the net effect of DOC production, absorption, and transport in the terrestrial component of the catchment, as well as processes of DOC release, adsorption, and decomposition in the stream channel.

The relatively low concentrations of DOC (4–8 mg·L^{-1}) observed in the streams draining catchments dominated by deep, well-drained soils in southern Québec is in accordance with other measurements of similar catchments (e.g. Clair and Freedman 1986; Dahm 1980; Eshleman and Hemond 1985; Foster and Grieve 1982; Hobbie and Likens 1973; McDowell and Likens 1988; Meyer and Tate 1983; Moore 1989; Naiman 1982; Schlesinger and Melack 1981). The higher DOC concentration in catchment 3 may be associated with generally shallower soils and steeper topography and the greater occurrence of coniferous vegetation, which has been shown to produce higher soil water DOC concentrations than deciduous forests (Cronan and Aiken 1985). The positive association between DOC and discharge in these catchments is also a commonly-observed phenomenon, associated with differences in DOC pathways during rainfall and runoff-generating events (Meyer and Tate 1983).

The higher concentrations of DOC (14–40 mg·L^{-1}) in streams draining catchments with a substantial component of wetland have also been found in other wetland streams (e.g. Brinson et al. 1980; Kerekes et al. 1986; Gorham et al. 1985; Grieve 1984; McKnight et al. 1985; Moore 1987a, b, c; Moore and Jackson 1989; Mulholland 1981; Mulholland and Kuenzler 1979; Naiman 1982; Schlesinger and Melack 1981; Urban et al. 1989). Several factors contribute to the high DOC concentrations in streams draining wetlands including the high rates of DOC production in organic soils. Wetlands are generally located close to the stream channel and water passes either through organic horizons, as overland flow or as subsurface flow through gleyed subsoil horizons with low DOC-adsorption capacities. Unlike streams draining non-wetland catchments, there is no strong association between DOC concentrations and discharge in wetland catchment streams, and a variety of positive and negative associations have been reported from other wetland catchments (e.g. Clair and Freedman 1986; Moore and Jackson 1989; Mulholland 1981). The poor linkage between discharge and DOC concentration adds to the difficulties in estimating DOC export from wetland catchments (Moore and Jackson 1989; Urban et al. 1989).

Sampling from spring to early winter in the southern Québec streams failed to reveal strong seasonal variations in DOC concentration, although DOC concentrations in three of the non-wetland streams suggested that in these catchments, production and transport of DOC is lower in summer than the fall and spring. These low summer concentrations may be related to the availability of fresh, decomposing plant tissues in the fall and spring in streams draining deciduous forests. In a British moorland stream, Grieve (1984) noted a sine wave pattern in the residuals of dissolved organic matter:discharge, with positive residuals in the late summer and fall, and negative in the early summer and winter.

Assuming an annual runoff of about 500 mm in southern Québec (Energy, Mines and Resources Canada 1974), export of DOC from these catchments ranges from about 1–3 g C·m^{-2}·yr^{-1} from the non-wetland catchments to 6 to 18 g C·m^{-2}·yr^{-1}m from the wetland catchments. Because of uncertainties in DOC:discharge relationships (especially in the wetland catchments) and DOC concentrations in spring runoff, these estimates should be treated with caution. The estimates, however, are in accord with DOC export estimates from other catchments (e.g. Mulholland and Kuenzler 1979; Urban et al. 1989).

The analysis of catchment variables revealed a strong positive association between the proportion of the catchment occupied by wetland and stream DOC concentration from spring to early winter. Although different wetland types produce different DOC concentrations (e.g. Moore 1987a, b, c; Moore and Jackson 1989; Urban et al. 1989) and differences in hydrologic pathways may also produce differences in the amount and concentration of DOC that reaches the stream channel, these appear to be of less importance than the area of the catchment occupied by wetland, in explaining differences in DOC concentrations of wetland streams.

The proportion of the variation in DOC concentration explained by percent wetland (r^2 varying between 0.26 and 0.67) is similar to that obtained by Rasmussen et al. (1989) in a model to predict lake colour from catchment variables. The model appears to work best during wet conditions (May and November), rather than during the summer, which was especially dry during 1988 in southern Québec. The standard errors of the regression model (ln DOC 0.09–0.18) are of the same order as the seasonal variation in DOC concentration within the streams, suggesting that the model could be used through the seasons. The results suggest that DOC concentrations and possibly DOC export from small catchments could be readily predicted from a simple, easily-determined cachment variable such as wetland area; regression coefficients and constants are similar for the five sampling dates. Because acid dissociation constants for DOC-rich waters fall in a narrow range, direct relationships between DOC and an easily-measured watershed attribute such as wetland area would be valuable in an examination of naturally-occurring acidity export to streams and lakes (Urban et al. 1989).

The results suggest that there are regional differences in the DOC:percent wetland relationship, as commonly occurs in such empirical models. In particular, differences in wetland type (e.g. fen, bog, or swamp) may be important, as well as the geochemical composition of the water, such as the specific conductance and Ca and Mg concentration, which may lower DOC concentrations through precipitation and adsorption (Wetzel and Otsuki, 1974). Also important are changes in land use, which affect the DOC-producing abilities of the vegetation and drainage of land for agriculture and thereby the pathways taken by water. This is particularly important in the heavily-utilised catchments in southern Québec, where many of the poorly drained soils and peats have been ditched. There is evidence of increases in stream DOC concentrations resulting from deforestation (e.g. Dahm 1980; Meyer and Tate 1983; Moore 1989; Tate and Meyer 1983), but little evidence for significant changes from wetland catchments that have been drained (e.g. Moore 1987b; Moore and Jackson 1989). Finally, it must be borne in mind that the results presented apply to small catchments (0.6–37.2 km^2) and that on a broader scale, area may also play a role in determining DOC concentrations and export, as has been shown for phosphorus (e.g. Prairie and Kalff 1986).

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References

Baril, R., and A. Mailloux. 1950. Étude pédologique des sols du comté de Chateauguay. Ministère de L'Agriculture, Québec.

Brinson, M. M., H. D. Bradshaw, R. N. Holmes, and J. B. Elkins. 1980. Litterfall, stemflow, and throughfall nutrient fluxes in an alluvial forest swamp. Ecology 61: 827–835.

Cann, D. B., P. Lajoie, and P. C. Stobbe. 1947. Soil Survey of Shefford, Brome, and Missisquoi Counties in the Province of Quebec. Department of Agriculture, Ottawa.

Clair, T. A., and B. Freedman. 1986. Patterns and importance of dissolved organic carbon in four acidic brownwater streams in Nova Scotia, Canada. Water Air Soil Pollut. 31: 139–147.

Cronan, C. S., and G. R. Aiken. 1985. Chemistry and transport of soluble humic substances in forested watersheds of the Adirondack Park, New York. Geochim. Cosmochim. Acta 49: 1697–1705.

Dahm, C. N. 1980. Studies on the distribution and fates of dissolved organic carbon. PhD. thesis, Oregon State University, Corvallis, Ore. 145 p.

Driscoll, C. T., R. D. Fuller, and W. D. Schecher. 1989. The role of organic acids in the acidification of surface waters in the eastern U.S. Water Air Soil Pollut. 43: 21–40.

Energy, Mines and Resources Canada. 1974. National Atlas of Canada. Macmillan Publ., Ottawa, Ont.

Engstrom, D. 1987. Influence of vegetation and hydrology on the humus budgets of Labrador lakes. Can. J. Fish. Aquat. Sci. 44: 1306–1314.

Eshleman, K. N., and H. F. Hemond. 1985. The role of organic acids in the acid-base status of surface waters at Bickford Watershed, Massachusetts. Water Resour. Res. 21: 1503–1510.

Foster, I. D. L., and I. C. Grieve. 1982. Short term fluctuations in dissolved organic matter concentrations in streamflow draining a forested watershed and their relation to the catchment budget. Earth Surface Processes and Landforms 7: 417–425.

Gorham, E., S. J. Eisenreich, J. Ford, and M. V. Santelman. 1985. The chemistry of bog waters. p. 339–363. *In* W. Stumm [ed.] Chemical processes in lakes. John Wiley and Sons, New York, NY.

Grieve, I. C. 1984. Concentrations and annual loading of dissolved organic matter in a small moorland stream. Freshwater Biol. 14: 533–537.

Hobbie, J. E., and G. E. Likens. 1973. Output of phosphorus, dissolved organic carbon, and fine particulate carbon from Hubbard Brook watersheds. Limnol. Oceanogr. 18: 734–742.

Kerekes, J., S. Beauchamp, R. Tordon, C. Tremblay, and T. Pollock. 1986. Organic versus anthropogenic acidity in tributaries of the Kejimkujik watersheds in western Nova Scotia. Water Air Soil Pollut. 31: 165–173.

Mailloux, A., and G. Godbout. 1954. Étude pédologique des sols des comtés de Huntingdon et Beauharnois. Ministère de L'Agriculture, Québec.

McDowell, W. H. 1985. Kinetics and mechanisms of dissolved organic carbon retention in a headwater stream. Biogeochemistry 1: 329–352.

McDowell, W. H., and G. E. Likens. 1988. Origin, composition, and flux of dissolved organic carbon in the Hubbard Brook valley. Ecol. Monogr. 58: 177–195.

McDowell, W. H., and T. Wood. 1984. Podzolization: soil processes control dissolved organic carbon concentration in stream water. Soil Sci. 133: 23–32.

McKnight, D. M., E. M. Thurman, R. L. Wershaw, and H. Hemond. 1985. Biogeochemistry of aquatic substances in Thoreau's Bog, Concord, Massachusetts. Ecology 66: 1339–1352.

Meyer, J. L., and C. M. Tate. 1983. The effects of watershed disturbance on dissolved organic carbon dynamics of a stream. Ecology 64: 33–44.

Moeller, J. R., W. Minshall, K. W. Cummins, R. C. Petersen, C. E. Cushing, R. J. Sedell, R. A. Larson, and R. L. Vannote. 1979. Transport of dissolved organic carbon in streams of differing physiographic characteristics. Org. Geochem. 1: 139–150.

Moore, T. R. 1985. The spectrophotometric determination of dissolved organic carbon in peat waters. Soil Sci. Soc. Am. J. 49: 1590–1592.

1987a. Patterns of dissolved organic matter in subarctic peatlands. Earth Surface Processes and Landforms 12: 387–397.

1987b. A preliminary study of the effects of drainage and harvesting on water quality in ombrotrophic bogs near Sept-Iles, Quebec. Water Res. Bull. 23: 785–791.

1987c. Dissolved iron and organic carbon in northern peatlands. Soil Sci. 145: 70–76.

1989. Dynamics of dissolved organic carbon in forested and disturbed catchments, Westland, New Zealand. I Maimai. Water Resour. Res. 25: 1321–1330.

Moore, T. R., and R. J. Jackson. 1989. Dynamics of dissolved organic carbon in forested and disturbed catchments, Westland, New Zealand. II Larry River. Water Resour. Res. 25: 1331–1339.

Mulholland, P. J. 1981. Organic carbon flow in a swamp-stream ecosystem. Ecol. Monogr. 51: 307–322.

Mulholland, P. J., and E. J. Kuenzler. 1979. Organic carbon export from upland and forested-wetland watersheds. Limnol. Oceanogr. 24: 960–966.

Naiman, R. J. 1982. Characteristics of sediment and organic carbon export from pristine boreal forest watersheds. Can. J. Fish. Aquat. Sci. 39: 1699–1718.

Prairie, Y. T., and J. Kalff. 1986. The effect of catchment size on phosphorus export. Water Resour. Bull. 22: 465–470.

Rasmussen, J. B., L. Godbout, and M. Schallenberg. 1989. The humic content of lake water and its relationship to watershed and lake morphometry. Limnol. Oceanogr. 34: 1336–1343.

SAS Institute Inc. 1985. SAS User's Guide: Statistics, 5th Ed. Cary, NC.

Schlesinger, W. H., and J. M. Melack. 1981. Transport of organic carbon in the world's rivers. Tellus 33: 172–187.

Suffet, I. H., and P. MacCarthy. 1989. Aquatic humic substances: influence on fate and treatment of pollutants. Advances in Chemistry Ser. 219, American Chemical Society, Washington, DC.

Tate, C. M., and J. L. Meyer. 1983. The influence of hydrologic conditions and successional state on dissolved organic carbon export from forested watersheds. Ecology 64: 25–32.

Thurman, E. M. 1985. Organic Chemistry of Natural Waters. Nijhoff/Junk Publishers, Boston, MA. p. 497.

Urban, N. R., S. E. Bayley and S. J. Eisenreich. 1989. Export of dissolved organic carbon and acidity from peatlands. Water Resour. Res. 25: 1619–1628.

Wetzel, R. G., and A. Otsuki. 1974. Allochthonous organic carbon of a marl lake. Arch. Hydrobiol. 73: 31–56.

Nitrogen Fixation by Epilithic Periphyton in Small Arctic Lakes in Response to Experimental Nitrogen and Phosphorus Fertilization

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Epilithic periphyton played a moderate role in the nitrogen budget of four small arctic lakes located at Saqvaqjuac, N.W.T. The acetylene reduction technique used to estimate nitrogen fixation indicated that periphyton in 0–2 m water depth were capable of fixing up to 0.86 mg $N \cdot m^{-2} \cdot h^{-1}$ during the period of active nitrogen fixation from July to September 1981. Far Lake was fertilized with Phosphorus for 3 yr and had a higher rate of fixation than did a P- and N-fertilized lake or a control lake, in agreement with similar studies on temperate and subarctic lakes. A numerical model based on incoming light was used to perdict the amount of nitrogen fixed in each study lake throughout the season. The calculated contribution of periphyton to the overall N budget for 1981 was 5% for the P- and N-fertilized lake, 16% for the control lake and 28% for the P-only fertilized lake.

Le périphyton épilithique a joué un rôle modéré dans le bilan azoté de quatre petits lacs arctiques situés à Saqvaqjuac (T. N.-).). La technique de réduction à l'acétylène utilisée pour évaluer la fixation de l'azote a montré que le périphyton dans des eaux dont la profondeur était entre 0 et 2 pouvait fixer jusqu'à 0,86 mg de $N \cdot m^{-2} \cdot h^{-1}$ pendant la période de fixation active de l'azote, de juillet à septembre 1981. Le lac Far a été fertilisé au P pendant trois ans et son taux de fixation était plus élevé que celui d'un lac fertilisé au P et à N ou celui d'un lac témoin, en accord avec des études similaires sur des lacs tempérés et subarctiques. Un modèle numérique fondé sur la lumière qui entre a été utilisé pour prévoir la quantité d'azote fixée dans chacun des lacs pendant toute la saison. L'apport calculé du périphyton au bilan global de N pour 1981 était de 5 % pour les lacs fertilisés au P et à N, de 16 % pour le lac témoin et de 28 % pour le lac fertilisé seulement au P.

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Biological fixation of atmospheric nitrogen is known to occur in temperate lakes and ponds in association with biota in both the water column and the sediments (Howard et al. 1970; Vanderhoef et al. 1974; Hooper–Ried and Robinson 1978; Flett et al. 1980; Loeb and Reuter 1981). Flett et al. (1980) found that the occurrence of planktonic nitrogen fixation was dependent upon the combination of nutrients applied to lakes of the Experimental Lakes Area (ELA) in northwestern Ontario. Nitrogen fixation occurred in all of the lakes studied whose N:P mass loading ratios were less than 10. Rates of N fixation by the periphyton were higher in lakes fertilized with P only than in lakes fertilized with N and P, N only, or in control lakes both at ELA (H. Holloway, Department of Fisheries and Oceans, Freshwater Institute, 501 University Cres., Winnipeg, Man. R3T 2N6) and the Kuokkel region of Sweden (Lundgren 1978).

As part of a study on the effects of eutrophication on arctic lakes, we wished to determine if N fixation was a significant part of lake N budgets, and whether fixation rates changed when lakes were fertilized with P or N and P. We have measured rates of N fixation by epilithic periphyton (for definition see Protasov 1982) using an acetylene reduction technique (Flett et al. 1976). Rates in three experimentally fertilized (P, or N and P) lakes were compared with those in a control lake (no fertilizer added). We predicted that the P-only fertilized lake would show the highest N fixation rates over the entire ice-free season.

We collected surface rocks colonized by periphyton from the sublittoral of each lake from July and August 1981. Nitrogen fixation rates were determined for the periphyton at ambient temperature and at a range of light intensities in the laboratory. We then calculated the amount of N fixed by each lake, for each day, during the ice-free season with a modified numeric model used for estimating primary production of phytoplankton (Fee 1984). Our experiments provided estimates of the parameters required to execute Fee's model. The model output provided estimates of the atmospheric N contribution to the total N budget for each lake.

Methods

Study Lakes

The four experimental study lakes (Far, P&N, Spring, and Jade) are located at Saqvaqjuac, N.W.T., a Fisheries and Oceans Canada research camp on the west coast of Hudson Bay (63°39′N, 90°39′W), 40 km north of Chesterfield Inlet. For a description of the area and history of the lakes, see Welch (1985). The study lakes are small (<8 ha; mean depth <4 m) and are typical of this region of the Canadian Precambrian granitic shield.

The lakes were sampled on 13 days over the period from 1 July to 28 August 1981. Far Lake, fertilized with P only (mean N:P = 5.1), was sampled on five occasions. P&N Lake

TABLE 1. Phosphorus and nitrogen loading rates, kg·ha^{-1} lake surface·yr^{-1}, for three Saqvaqjuac lakes, mean of 1979–81 (from Welch and Legault 1986).

Lake	N (natural)	N (fertilizer)[a]	Total N	P (natural)	P (fertilizer)[b]	Total P	N:P
Spring	6.69	0.0	6.69	0.114	0.0	0.114	59.0
Far	5.79	0.0	5.79	0.140	1.0	1.140	5.1
P&N	4.58	11.6	16.18	0.106	1.0	1.106	14.6

[a] As $NaNO_3$.

[b] As H_3PO_4.

was fertilized with both P and N (mean N:P = 14.6) and was sampled three times. Spring Lake was not fertilized (mean N:P = 59) and was sampled four times. Jade Lake, fertilized with P and N, was sampled on one occasion. Nutrient loading rates for three lakes are given in Table 1.

The 0–2.0 m zone of each lake is paved with rocks ranging from coarse gravel to small boulders, providing a nearly continuous surface upon which epilithic periphyton grows. Nitrogen-fixing, heterocystous, blue-green algae are common components of this community. Planktonic N-fixers are present in very low numbers for only a few weeks during the open-water season (Welch et al. 1989).

Acetylene Reduction by Epilithic Periphyton

In vitro periphytic N fixation was measured, using a modification of the acetylene reduction technique described by Flett et al. (1976). In an acetylene saturated environment, nitrogen-fixing species of algae reduce acetylene (C_2H_2) to ethylene (C_2H_4)(Flett et al. 1976). Ethylene production is proportional to the amount of dinitrogen fixed, where moles N_2 fixed = 0.33 × moles C_2H_4 produced (Flett et al. 1976). On each experimental day, one lake was sampled, and we selected 15–18 flat rocks ranging from 30 to 150 cm^2 in area. Rocks were then placed, individually, in clear acrylic 850 mL chambers (Fig. 1). Locations (20–100 m^2) were chosen at each study lake to maximize the number of rocks available for each experimental run and to minimize the degree of lake bottom slope. The total depth range over the course of all experiments was 0.2–0.6 m; however, within each experiment the depth varied only ± 0.1 m. Forceps were used to place the rocks into a submerged chamber to reduce loss of material. The periphyton in our study lakes is tightly attached and we estimate that little material loss occurred during the handling process.

Within 60 min of rock collection the chambers were filled with acetylene-saturated water (2 L welder grade acetylene and 18 L lake water obtained from a nearby lake), and held for 4 h in a graded light intensity incubator at the current lake temperature. On each day, a total of 16 chambers were run, two at each of eight light intensities (16, 26, 46, 94, 163, 332, 484, and 557 $\mu E \cdot m^{-2} \cdot s^{-1}$) measured by a flatplate Licor 185 quantum light meter. The light intensities were established through attenuation by distance from a light source within a covered incubator. The typical midsummer daily range of surface irradiance is 0–2000 $\mu E \cdot m^{-2} \cdot s^{-1}$. The mean 24 h light intensity at 0.25 m lake depth is 550 $\mu E \cdot m^{-2} \cdot s^{-1}$ on 15 July, decreasing to 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ on 15 September.

Sample preparation

Samples were taken for ethylene production, initially from the acetylene-water mixture, and after incubation and mixing, from each chamber using 50 mL syringes through a serum-stopper port located on the side of the chamber. Each syringe was filled with 30 mL of sample followed by 20 mL air and allowed to reach room temperature. The sample was then shaken vigorously for 1 min to equilibrate the liquid and gas phases. The remainder of the sample analysis, including the preparation of the ethylene standards, followed the method of Flett (1977). Briefly, we filled a 10 mL serum bottle with lake water and fitted it with a serum-stopper. We then injected the bottle with the gas sample which displaced the water via a vent needle. Duplicate 0.2 mL gas aliquots were then withdrawn and the ethylene content assayed by gas chromatographic analysis performed on a hydrogen flame ionization detector (Pye Unicam model 104) equipped with a phenylisocyanate/Porasil C packing at 45°C.

To compare rates of N fixation between lakes, we wished to express them as a function of periphyton biomass. Biomass determination by chlorophyll *a* measurements, however, was very time-consuming, requiring scrubbing all surfaces of each rock. In P&N Lake, over the range of rock sizes used, rock area and chlorophyll *a* were found to be linearly correlated ($r^2 = 0.61$, $n = 52$, $P = 0.0001$). Because area was the simpler measurement, all N fixation values were expressed as a function of projected rock area, and were also normalized per unit area.

Computer modelling

We calculated whole-lake nitrogen loading by the N-fixing periphyton based on N fixation rates obtained in the period from 1 July to 28 August. To obtain whole-lake N fixation we applied a modification of a numeric model developed by Fee (1984) for estimation of phytoplankton primary production to our data. This model is appropriate because the N fixing organisms in these lakes are photosynthetic and showed a strong response to incident solar radiation (Fig. 2). The model is used to calculate N fixation at a given irradiance using the photosynthetic parameters α and P^B_m which in turn are calculated from the incubation data. The parameters α and P^B_m are commonly used to empirically describe the photosynthesis-light response curve for phytoplankton (Jassby and Platt 1976; Fee 1984). Alpha (α) describes the slope of the light saturation curve at low light levels and P^B_m describes the photosynthetic optimal irradiance (Jassby and Platt 1976). In this study, α and P^B_m are not used to represent carbon fixation but the amount of nitrogen fixed, normalized per unit area.

Using daily Saqvaqjuac incident solar radiation data obtained every 30 min, and biweekly light extinction data to calculate light at different depths (Welch et al. 1987), N fixation totals were determined (0–2.0 m contour) for each 0.5 m depth for each day and summed over the period of active N fixation (12 July–22 September) to obtain the total contribution of periphytic N fixation to each lake. This total was then expressed as a percentage of the lake's annual nitrogen budget (Welch and Legault 1986). No fixation was assumed to occur after 22 September, the approximate time of lake freeze-up.

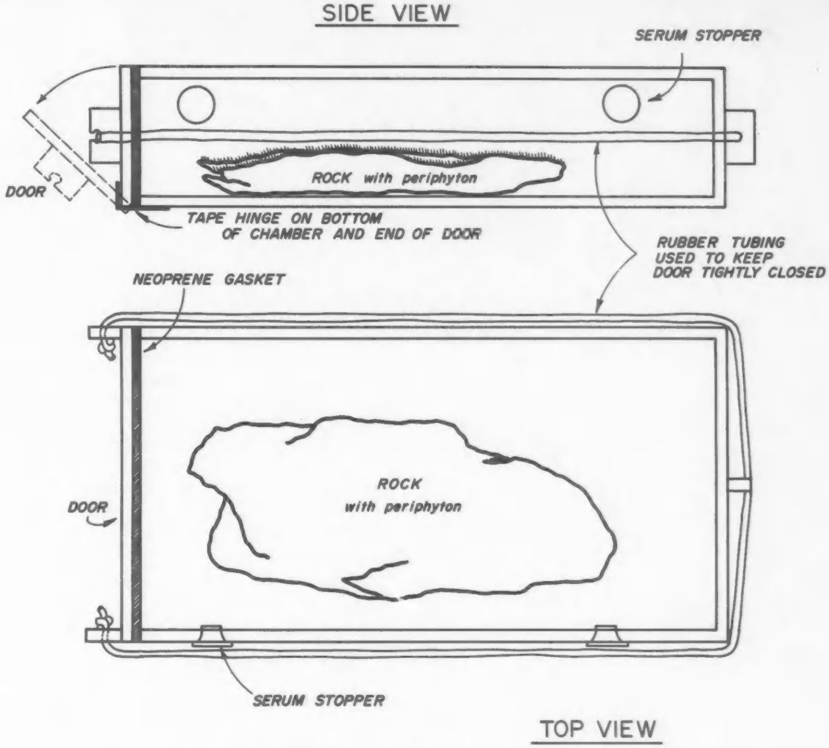
SIDE VIEW
SERUM STOPPER
DOOR
ROCK with periphyton
TAPE HINGE ON BOTTOM OF CHAMBER AND END OF DOOR
RUBBER TUBING USED TO KEEP DOOR TIGHTLY CLOSED
NEOPRENE GASKET
DOOR
ROCK with periphyton
SERUM STOPPER
TOP VIEW

FIG. 1. Chambers used to incubate rock periphyton.

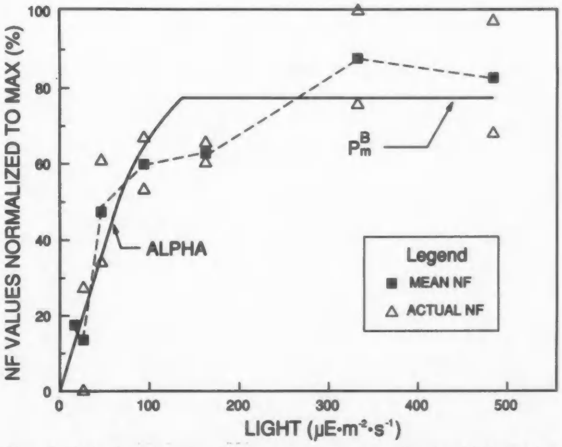

FIG. 2. Actual and mean nitrogen fixation normalized (100%) to the maximum; versus incubator light values for P&N Lake (3 August 1981). Parameters alpha and P_m^B are shown fitted to actual data set.

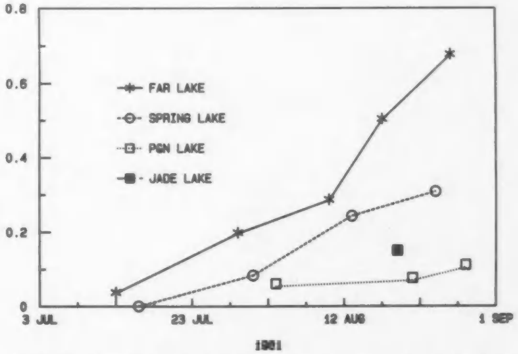

FIG. 3. Mean nitrogen fixation rates for all lakes throughout the 1981 sampling period.

Results and Discussion

Nitrogen Fixation

The dinitrogen:acetylene (N_2:C_2H_4) molar ratio of 0.33 used in this study represents the theoretical chemical conversion factor and has been applied elsewhere (Flett et al. 1976; Reuter et al. 1983b). We did not perform nitrogen-15 comparisons to test this ratio, and therefore it may not be the correct conversion required for our epilithic periphyton N fixation estimates (Graham et al. 1980). Using the mean molar ratio of $N_2:0.13 \times C_2H_4$ as applied to phytoplankton in a series of lakes in northwest Ontario (Graham et al. 1980) would result in a reduction of estimated N fixation rates for our lakes by a factor of 0.394.

No benthic N fixation was detected until July (3 wk after ice-off). From 16 July to 28 August, N fixation by epilithiphyton was detectable in all four study lakes. Mean N fixation rates differed over the sampling period among lakes (Fig. 3), with the highest rates occurring in Far Lake, fertilized with P only.

TABLE 2. Maximum N-fixation rates for epilithic periphyton as determined by the acetylene reduction method.

Lake	Sampling date	Maximum N_2^- fixation rate (mg N·m^{-2}) ·h^{-1}	% total annual N-budget (Natural and artificial loading)	Z_{mean} (m)	Artificial loading (g·m^{-2}·yr^{-1})	Reference
Hymenjaure	July 1973	0.40	17	1.3	0.25 P	Persson et al. (1977); Lundgren (1978)
Hymenjaure	July 1974	1.66	48	1.3	0.31 P	Persson et al. (1977); Lundgren (1978)
Hymenjaure	Aug. 1975	0.05	0.6	1.3	0.32 P,4.3 N	Persson et al. (1977); Lundgren (1978)
Stugsjon	July 1974	0.09	6	1.2	—	Persson et al. (1977); Lundgren (1978)
Stugsjon	Aug. 1975	0.17	7	1.2	—	Persson et al. (1977); Lundgren (1978)
Crater	Aug.–Sept. 1979	0.10	—	325	—	Loeb and Reuter (1981)
Tahoe	Aug.–Sept. 1979	0.14	—	313	—	Loeb and Reuter (1981)
Fallen Leaf	Aug.–Sept. 1979	0.04	—	53	—	Loeb and Reuter (1981)
Donner	Aug.–Sept. 1979	0.12	—	20	—	Loeb and Reuter (1981)
Castle	Aug.–Sept. 1979	0.11	—	11	—	Loeb and Reuter (1981)
L226NE	Aug. 1976	1.87	—	5.7	0.59 P,3.16 N	Holloway (unpubl. data)
P&N	Aug. 1981	0.26	5	3.3	0.10 P,1.0 N	Present study
Far	Aug. 1981	0.95	28	3.6	0.10 P	Present study
Spring	Aug. 1981	0.56	16	2.7	—	Present study

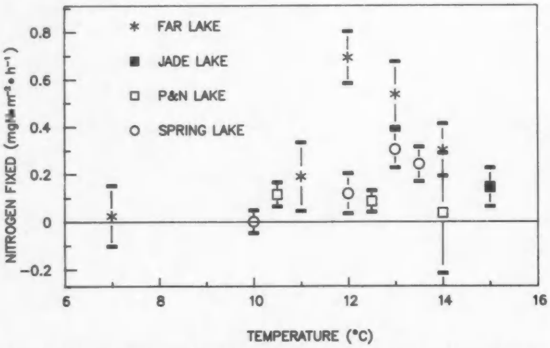

FIG. 4. Mean nitrogen fixation versus lake temperature for all lakes (1981); bars indicate 1 SD of individual estimates.

Nitrogen fixation was determined on one day only in a fourth study lake, Jade Lake (P and N fertilized), and was intermediate between rates obtained for P&N (P and N fertilized) and Spring (not fertilized) Lakes. Because this lake was only sampled once we did not attempt to estimate total annual N fixation.

The periphyton fixation rates of Saqvaqjuac lakes are similar to rates in high-altitude, deep lakes in northern California (Loeb and Reuter 1981) where the overall ambient summer temperature regime is similar, but they are less than rates in the subarctic Kuokkel lakes (Lundgren 1978).

Schlinder (1977) showed that N-deficient lakes, particularly those artificially enriched with P, exhibit higher N fixation rates than N-sufficient lakes (Table 2), being able to increase total nitrogen (TN) stocks gradually by N fixation. Likewise at Saqvaqjuac, Far Lake (P-only) had the highest N fixation rates, followed by Spring Lake (control) and P&N Lake (10:1 N and P enriched). No net increase in annual concentrations of total dissolved nitrogen or TN was found in response to our artificial nutrient loading (H. E. Welch, unpubl. data). For the P-enriched lakes, maximum N fixation rates were lower at Saqvaqjuac than at ELA and the Kuokkel area (Table 2). Unfertilized Spring Lake exhibited higher maximum N fixation rates than Stugsjon Lake or the northern California lakes (Table 2). This may reflect differences in nutrient levels, N:P ratios, temperature and mean depth (see below).

Despite considerable variability, there was a clear relationship between light and N fixation (Fig. 2), with increasing fixation up to 500 μE·m^{-2}·s^{-1}. Although Reuter et al. (1983a) found that periphytic nitrogenase activity reached maximum values at 25 μE·m^{-2}·s^{-1} and remained constant above that, Lundgren (1978) and Flett et al. (1980) found positive correlations between N fixation and light for epilithic and planktonic algae, respectively.

Nitrogen fixation was negligible below 10°C, appeared to peak at 12°C–13°C, and decreased above 13°C (Fig. 4). Reuter et al. (1983a) and Goering and Ness (1964) found increasing rates of aquatic N fixation with increasing temperature up to 30°C. The Saqvaqjuac lakes reach a summer maximum in mid-August, 6 wk after ice-out, followed by a rapid cooling, yet N fixation rates were still increasing at the end of August when lake temperatures had begun to drop (Welch et al. 1987, Fig. 3). Thus the apparent effects of temperature (Fig. 4) may be an artifact caused by the steady increase in N fixation throughout a season of rising, then falling temperatures.

There was no linear correlation ($P = 0.19$, $N = 161$) between TDN and N fixation, although there was an increase in N fixation with decreasing dissolved inorganic N (DIN) for Far Lake. In 1981, Far Lake DIN dropped rapidly from about 550 mg·m^{-3} at the onset of the growing season to <230 mg·m^{-3} by mid-July, remaining low until freeze-up. DIN values were always higher than 300 mg·m^{-3} in P&N Lake (unpubl. data). Most studies have shown that increased heterocystous nitrogenase activity occurs in response to low (<200 mg·m^{-3}) DIN concentrations (Stewart et al. 1971; Persson et al. 1977), although Reuter et al. (1983a, b) found no such response in Tahoe and Castle lakes where DIN concentrations are extremely low (<10 mg·m^{-3}) throughout the year.

Our data for Spring and Far lakes show that N fixation rates increase as lake total N to total P ratios decrease from 67 down to 12 (Fig. 5). We are not aware of any previous studies showing this relationship. Flett et al. (1980) showed that lakes with total N to total P ratios <10 exhibit planktonic N fixation, but no benthic data are available.

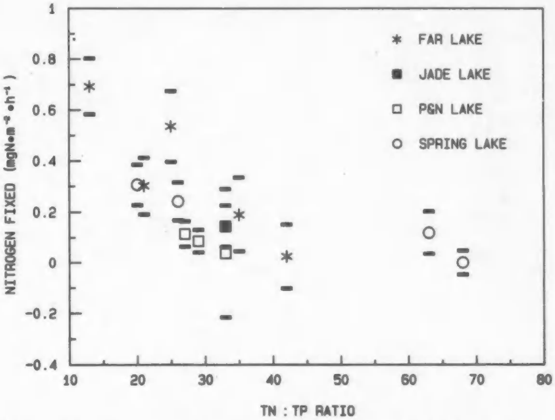

FIG. 5. Mean nitrogen fixed versus TN:TP ratio (1981); bars indicate 1 SD of individual estimates.

TABLE 3. Nitrogen budget for three small lakes at Saqvaqjuac, N.W.T., with special reference to nitrogen fixation, 1981. Nitrogen loading data from Welch and Legault (1986).

Lake	Area (ha)	% of lake area ≤ 2 m	Annual NF 0–2 m,kg	Annual external N loading to lake (kg)	% of nitrogen Budget from NF
Far	3.70	41	6.66	2.41	28
Spring	6.94	50	7.74	47.5	16
P&N	7.09	47	5.15	32.3[a]	16
				78.0[b]	—
				110.3[c]	5

[a] Natural N loading.
[b] Fertilizer N loading.
[c] Total N loading.

Epilithic periphyton samples taken from Saqvaqjuac lakes in July 1982 showed that three of the four dominant N fixing genera of algae present (*Calothrix*, *Dichothrix*, and *Homeothrix*) were the same in P&N Lake and Far Lake (H. Kling, Department of Fisheries and Oceans, Freshwater Institute, 501 University Cres., Winnipeg, Man. R3T 2N6, pers. comm.). Thus generic composition did not appear to substantially differ between the P-treated and P- and N-treated lakes after 4 yrs of perturbation. These lakes may be capable of rapidly increasing N fixation rates under higher levels of P because N fixing species are already present in the existing algal community.

Nitrogen fixation model

Our objective was to use numerical modelling to estimate the contribution of benthic periphyton to the nitrogen budget of the study lakes throughout the entire ice-free season. We chose Fee's (1984) primary production model because the response of fixation rates to incubator irradiance in our study can be described by a mathematical function similar to that used by Fee to describe the response of photosynthetic carbon fixation to irradiance. Flett (1977) used a similar approach to model the light response of planktonic N fixing algae in small lakes in northwest Ontario. Our N fixing and light data allowed us to estimate the model parameters, α and P_m^B, and we used local daily irradiance values to obtain N fixation estimates, expressed per square metre for each lake depth (Fee 1984). The optimal substrate for algal colonization is in less than 2 m water depth so we used the 2 m contour as the lower limit for periphytic nitrogenase activity. In this way, we attribute 28, 16, and 16% of the total annual natural N-loading to N fixation by the rock periphyton for Far, Spring, and P&N lakes, respectively (Table 3). There may have been some N fixation occurring below 2 m depth but we have not attempted to estimate this. Sediment accumulation increases below 2 m (depth of maximum ice thickness) and the periphyton-covered, rocky substrate which characterizes the 0–2 m depth range can no longer be observed (H. E. Welch, pers. observ). This means, however, that the percentage contributions by N fixation to total N budgets given in Tables 2 and 3 are probably underestimates.

Estimates of N fixation beyond our sampling period our sampling period (31 August–22 September) were made by linear extrapolation from the last sampling date to the estimated date of zero fixation (22 September, onset of ice formation).

A comparison of estimates of total N fixed per annum was possible only between the lakes of the Kuokkel region of Sweden (Lundgren 1978; Persson et al. 1977) and the present study (Table 2). The percent of the total annual N budget which is due to N fixation is highest for the P-only fertilized lakes, and lowest for the P- and N-fertilized lakes. The periphytic contribution of N fixed to Lake Hymenjaure was greater than in Far Lake, due both to its higher P:N loading rate and shallower depth.

In summary, our study shows that the periphyton of small arctic lakes can fix a significant fraction of the total lake nitrogen budget, and the proportion increases with an increase in P-loading and a decrease in the N:P ratio. These findings are consistent with other work (e.g. Lundgren 1978) and demonstrate that arctic periphytic N fixing communities are capable of adapting to changes in lake nutrient status.

In this study N fixation rates are light dependent and can be easily modelled using a numeric relationship developed for phytoplankton photosynthesis. This allows for the application of a generalized model to N fixation estimates. Future studies in which N fixation estimates of benthic periphyton are made on an infrequent basis can be used in conjunction with incident light data and lake extinction coefficients to calculate the contribution of atmospheric N to the overall lake budget.

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References

FEE, E. J. 1984. Freshwater Institute primary production model user's guide. Can. Tech. Rep. Fish. Aquat. Sci. 1328: v + 36 p.

FLETT, R. J. 1977. Nitrogen fixation in Canadian Precambrian Shield lakes. Ph.D. thesis, University of Manitoba, Winnipeg, Man. 197 p.

FLETT, R. J., R. D. HAMILTON, AND N. E. R. CAMPBELL. 1976. Aquatic acetylene-reduction techniques: solutions to several problems. Can. J. Microbiol. 22: 43–51.

FLETT, R. J., D. W. SCHINDLER, R. D. HAMILTON, AND N. E. R. CAMPBELL. 1980. Nitrogen fixation in Canadian Precambrian Shield lakes. Can. J. Fish. Aquat. Sci. 37: 494–505.

Goering, J. J., and J. C. Ness. 1964. Nitrogen fixation in two Wisconsin lakes. Limnol. Oceanogr. 9: 530–539.

Graham, B. M., R. D. Hamilton, and N. E. R. Campbell. 1980. Comparison of the nitrogen-15 uptake and the acetylene reduction methods for estimating the rates of nitrogen fixation by freshwater blue-green algae. Can. J. Fish. Aquat. Sci. 37: 488–493.

Hooper-Ried, N. M., and G. G. C. Robinson. 1978. Seasonal dynamics of epilithic algal growth in a marsh pond: composition, metabolism, and nutrient availability. Can. J. Bot. 56: 2448.

Howard, D. L., J. I. Frea, R. M. Pfister, and P. R. Dugan. 1970. Biological nitrogen fixation in Lake Erie. Science (Wash. DC) 169: 61–62.

Jassby, A. D., and T. Platt. 1976. Mathematical formulation of the relationship between photosynthesis and light for phytoplankton. Limnol. Oceanogr. 21: 540–547.

Loeb, S. L., and J. E. Reuter. 1981. The epilithic periphyton community: a five-lake comparative study of community productivity, nitrogen metabolism and depth distribution of standing crop. Verh. Int. Vere. Limnol. 21: 346–352.

Lundgren, A. 1978. Nitrogen fixation induced by phosphorus fertilization of a subarctic lake. *In* V. Granhall [ed.] Environmental role of nitrogen-fixing blue-green algae and asymbiotic bacteria. Swedish Natural Science Research Council. Ecol. Bull. (Stockholm) 26: 52–59.

Persson, G., S. K., Holmgren M. Jansson, A. Lundgren, B. Nyman, D. Solander, and C. Anell. 1977. Phosphorus and nitrogen and the regulation of lake ecosystems. Experimental approaches in subarctic Sweden. *In* Proc. Circumpolar Conference on Northern Ecology, Sept. 1975. III: p. 1–19. Ottawa: National Research Council of Canada.

Protasov, A. A. 1982. Periphyton:terminology and basic definitions. Hydrobio. J. 18: 1–5.

Reuter, J. E., S. L. Loeb, and C. R. Goldman. 1983a. Nitrogen fixation in oligotrophic Lake Tahoe. *In* R. G. Wetzel [ed.] Periphyton in Freshwater Ecosystems. Dr. W. Junk Publishers, the Hague, Netherlands.

Reuter, J. E., S. L. Loeb, R. P. Axler, R. G. Carlton, and C. R. Goldman. 1983b. Transformations of nitrogen following an eplimnetic nitrogen fixation in Castle Lake. CA. 1. Epilithic periphyton responses. Arch. Hydrobiol. 102: 425–433.

Schindler, D. W. 1977. Evolution of phosphorus limitation in lakes. Science (Wash., DC) 195: 260–262.

Stewart, W. D. P., T. Magne, G. P. Fitzgerald, and R. H. Burris. 1971. Nitrogenase activity in Wisconsin lakes of differing degrees of eutrophication. New Phytol. 70: 497–509.

Vanderhoef, L. N., C-Y. Huong, and R. Musil. 1974. Nitrogen fixation (acetylene reduction) phytoplankton in Green Bay, Lake Michigan, in relation to nutrient concentrations. Limnol. Oceanogr. 19: 119–125.

Welch, H. E. 1985. Introduction to limnological research at Saqvaqjuac, Northern Hudson Bay. Can. J. Fish. Aquat. Sci. 42: 494–505.

Welch, H. E., and J. A. Legault. 1986. Precipitation chemistry and chemical limnology of fertilized and natural lakes at Saqvaqjuac, N.W.T. Can. J. Fish. Aquat. Sci. 43: 1104–1134.

Welch, H. E., J. A. Legault, and M. A. Bergmann. 1987. Effects of snow and ice on the annual cycles of heat and light in Saqvaqjuac lakes. Can. J Fish. Aquat. Sci. 44: 1451–1461.

Welch, H. E., J. A. Legault, and H. Kling. 1989. Phytoplankton, nutrients and primary production in fertilized and natural lakes at Saqvaqjuac, N.W.T. Can. J. Fish. Aquat. Sci. 46: 90–107.

Effect of an External Tag on Growth of Sablefish (*Anoplopoma fimbria*), and Consequences to Mortality and Age at Maturity

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Tagged juveniles from a strong year-class of sablefish (*Anoplopoma fimbria*) were sampled for 9 yr Tagged females were approximately 6 cm larger than untagged females at 50% maturity. Males showed little difference because of their slower growth rate. Untagged males and females matured one and 2 yr earlier than tagged males and females. From this same year-class length-at-age of untagged males and females was significantly larger than for tagged and recaptured fish. Untagged males and females were 6.5 and 8 cm larger than tagged fish by age seven. Smaller tagged fish were recaptured at lower rates than larger tagged fish, indicating that slower growth reduced recapture percentage, which we believe indicates a higher mortality rate. Because smaller size was associated with increased mortality and tagged fish grew slower than untagged fish, we concluded that tagged fish had higher rates of mortality than untagged fish. The increased age and size at 50% maturity and the increased juvenile mortality indicated the potential response of a population of sablefish and possibly other species to a reduction in individual fish growth. If our results apply to other species and other types of tags, investigators should be cautious in extrapolating from tagged to untagged populations.

Pendant neuf ans, on a échantilloné de juvéniles étiquetés de morue charbonnière (*Anoplopoma fimbria*) appartenant à une abondante classe d'âge. À un taux de maturité de 50 %, les femelles étiquetées mesuraient environ 6 cm de plus que les femelles non étiquetées. Les mâles n'étaient que légèrement différents à cause de leur faible taux de croisssance. Les mâles et les femelles non étiquetés ont respectivement atteint la maturité un an et deux ans plus tôt que les mâles et les femelles étiquetés. De plus, la longueur des mâles et des femelles non étiquetés à un âge donné était nettement supérieure à celle des poissons étiquetés et recapturés. À sept ans, les mâles et les femelles non étiquetés mesuraient respectivement 6,5 et 8 cm de plus que les poissons étiquetés. Les petits poissons étiquetés ont été recapturés à un plus faible taux que les gros poissons étiquetés; ceci révèle qu'une plus lente croissance réduit le pourcentage de recapture qui, selon les auteurs, montre un taux de mortalité plus élevé. Étant donné qu'une petite taille était liée à un taux de mortalité plus élevé. Étant donné qu'une petite taille était liée à un taux de mortalité plus élevé et que les poissons étiquetés montraient un plus faible taux de croissance que les poissons non étiquetés, les auteurs formulent la conclusion que les poissons étiquetés sont l'objet d'un taux de mortalité plus élevé que les poissons non étiquetés. Un âge plus avancé et une taille plus grande à un taux de maturité de 50 % et le taux de mortalité plus élevé des juvéniles révèlent la réaction potentielle d'une population de morue charbonnière et peut-être d'autres espèces à une baisse du taux de croissance individuel. Si les résultats de l'étude s'appliquent à d'autres espèces et à d'autres genres d'étiquettes, les chercheurs devraient être prudents dans l'extrapolation des résultats obtenus de poissons étiquetés à des poissons non étiquetés.

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Sablefish (*Anoplopoma fimbria*) are a long-lived fish (Beamish and Chilton 1982) that are captured off the west coast of North America in traps set on the bottom. Currently, the sablefish fishery is the second most important west coast groundfish fishery. Most commercially caught sablefish range in age from 4 to 35 yr (McFarlane and Beamish 1983) but can be as old as 70 yr. Males in the commercial fishery range in size from 38 to 91 cm but less than 10% exceed 70 cm. Females range from 30 to 114 cm and more than 55% are larger than 70 cm. Most fish in the commercial fishery range in length from 60 to 75 cm. Length at 50% maturity was estimated to be 52 cm for males and 58 cm for females (Mason et al. 1983). Parks and Shaw (1983) reported lengths at 50% maturity in U.S. waters of 49.7 and 54.9 cm for males and females, respectively. We examined the general problem of accuracy of estimates of age and size at 50% maturity for sablefish by tagging juveniles from a strong year-class produced in 1977. We found that tagging appeared to be affecting the growth of sablefish. In this paper we examine the difference in growth of tagged and untagged fish from the same year-class and what consequences this has on mortality, age, and size at 50% maturity.

The relationship of age or size at sexual maturity is important in the understanding of the productivity of a population, particularly if changes in the relationship occur during exploitation. Changes in age or size at 50% maturity can indicate changes in the instantaneous population growth rate and can affect the optimal fishing mortality rate (Welch and Foucher 1988). If a relationship exists between size at 50% maturity and recruitment (Sinclair et al. 1982), an accurate estimate of this size is needed to understand the effect of exploitation on reproductive potential or for calculations of partial recruitment. Our study indicates that the tagged fish are not representative of the biology of untagged fish. By reducing the amount of growth, we demonstrated how sablefish could respond to changes that caused slower growth.

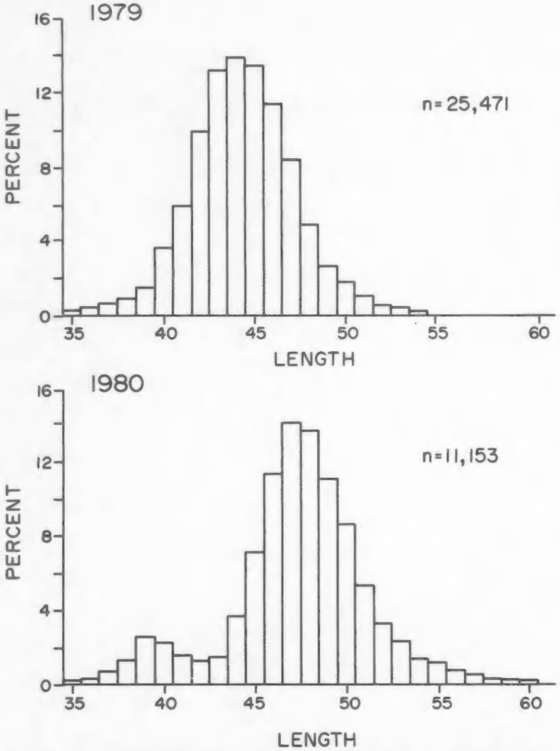

FIG. 1. Length frequency of juvenile sablefish (1977 year-class) tagged and released in "inside" waters, 1979 and 1980. The 1980 sample includes a small number of the 1978 year-class (mode at 39 cm). The 1978 year-class was identified using the modal analysis of Foucher and Fournier (1982) and excluded from the analysis.

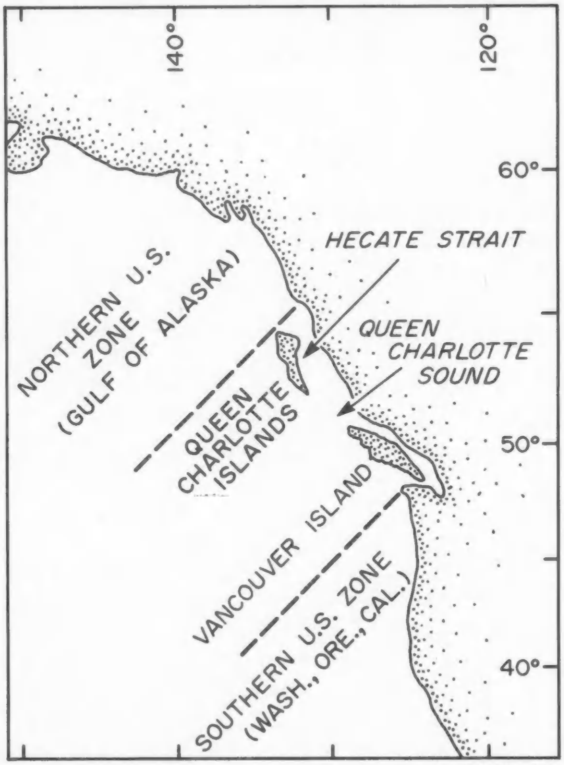

FIG. 2. Major areas referred to in this study. Hecate Strait, Queen Charlotte Sound, and coastal inlets are considered to be "inside" waters. Areas off the west coast of Queen Charlotte Island, Vancouver Island, Queen Charlotte Sound, and off the northern and southern coast of the United States are considered "outside" waters.

Materials and Methods

All juveniles were captured in Hecate Strait and Queen Charlotte Sound with trawl nets, tagged, and released at the capture site. A Floy FD-68 anchor tag was applied according to the procedures described in Beamish et al. (1979) and Beamish and McFarlane (1988). Juvenile sablefish from the 1977 year-class were captured in 1979 and 1980. A small number of juvenile fish were sampled each year for fork length, otoliths, sex, and maturity. Most (94%) tagged fish were recaptured in commercial sablefish fisheries that used traps and longlines. The remainder were caught in trawl nets. Age was determined according to the procedures of Beamish and Chilton (1982). Sablefish were collected from the commercial trap fishery each year from 1979–87 to determine age composition. Discards of juvenile sablefish were monitored in the commercial trawl fishery annually.

This study examined the biology of tagged and untagged fish from one year-class. Conclusions depend on the assumption that tagged fish were from one year-class, the 1977-year class. The lengths (Fig. 1) from the sample tagged in 1979 were normally distributed and almost all of the fish examined for age were estimated to be from the 1977 year-class (age 2+). In 1980, 86% of the fish examined for age were estimated to be age 3+. The smallest fish were from the 1978 year-class, (modal analysis, Foucher and Fournier 1982), and were eliminated from the analysis. The larger fish could have come from year-classes prior to 1977, but because they were not present in the 1979 sample, it was assumed that they were from the 1977 year-class. In addition, to ensure that the earlier year-classes were not included in the analysis, fish larger than 50 cm were excluded. The length frequency, the ages of fish sampled, and the exclusion of fish at the extremes of the length frequency distribution, all indicated that the fish used in this analysis were from the 1977 year-class.

Male sablefish grow more slowly than females, beginning at an average size of 45 cm (McFarlane and Beamish 1983). This difference in growth rate between males and females is considered in all of our analyses. Mean size and age at 50% maturity was determined using probit analysis.

Recaptured fish were separated by gear type, depth, and area. To facilitate reporting Hecate Strait, Queen Charlotte Sound, and adjacent coastal inlets were considered to be "inside waters." Areas off the west coast of Queen Charlotte Island, Vancouver Island, Queen Charlotte Sound, and off the coast of the United States were considered to be "outside" waters (Fig. 2).

Results

Numbers Tagged and Recaptured

In 1979 and 1980, 25 471 and 11 153 juvenile sablefish were tagged and released, respectively. As of December 31, 1987,

TABLE 1. Mean length of juvenile sablefish (1977 year-class) by recovery area, 1979–87 (total number of fish in parenthesis).

Release year	Recovery area	1979 M	1979 F	1980 M	1980 F	1981 M	1981 F	1982 M	1982 F	1983 M	1983 F	1984 M	1984 F	1985 M	1985 F	1986 M	1986 F	1987 M	1987 F
1979	Inside	45.7(3)	48.0(4)	46.0(37)	46.8(56)	49.8(7)	52.5(8)	53.9(5)	57.3(10)	50.4(2)	57.3(5)	54.6(4)	—	—	—	61.3(2)	70.7(3)	—	—
	Outside	—	—	50.1(5)	49.2(5)	51.8(14)	55.6(21)	55.5(21)	57.2(20)	56.2(10)	60.9(16)	56.3(8)	62.1(5)	58.2(2)	53.2(4)	58.2(5)	65.9(2)	62.8(2)	71.5(7)
1980	Inside	—	—	—	51.5(6)	49.7(16)	54.7(15)	52.3(9)	50.1(3)	53.3(5)	56.4(1)	51.3(3)	62.4(2)	54.2(1)	—	—	65.4(1)	—	—
	Outside	—	—	—	—	52.4(8)	54.9(15)	53.2(10)	55.8(18)	55.2(15)	57.4(14)	54.4(4)	60.3(5)	69.9(4)	59.6(4)	59.3(5)	64.3(2)	66.8(2)	77.1(2)

TABLE 2. Mean lengths of tagged and untagged fish from the 1977 year-class tagged in 1979 and 1980 and recaptured in outside waters.

Age	Sample year	Untagged Male (n)	Untagged Female (n)	Tagged Male (n)	Tagged Female (n)
3	1980	48.7 (101)	49.8 (165)	[a]50.1 (5)	49.2 (5)
4	1981	54.9 (38)	61.3 (201)	[a]51.8 (14) [b]52.4 (8)	55.6 (21) 54.9 (15)
5	1982	57.3 (127)	62.4 (292)	[a]55.5 (21) [b]53.2 (10)	57.2 (20) 55.8 (10
6	1983	58.0 (100)	65.0 (237)	[a]56.2 (10) [b]55.2 (15)	60.9 (16) 57.4 (14)
7	1984	61.7 (62)	68.9 (188)	[a]56.3 (8) [b]54.4 (4)	62.1 (5) 60.3 (5)
8	1985	61.0 (81)	71.1 (139)	[a]58.2 (2) [b]54.9 (4)	53.2 (4) 59.6 (4)
9	1986	61.9 (19)	72.3 (34)	[a]58.2 (5) [b]59.3 (5)	65.9 (2) 64.3 (2)

[a] Recaptures from 1979 releases.
[b] Recaptures from 1980 releases.

577 tagged fish (1.6%) were recaptured. A total of 215 were recaptured from inside waters and 275 were recaptured from outside waters (Table 1). Eighty-seven tagged juveniles were recovered but lacked any recovery information and were not included in our analysis. The number of recaptures in the inside waters declined rapidly and, by 1982, the majority of recaptures were made in the outside area (inside 39%; outside 61%).

Length Comparisons

The ratio of males to females recaptured in inside waters and outside waters was almost equal. The mean length-at-age of males was smaller than that of females in both inside and outside areas (ANOVA $P \leqslant 0.05$; $F = 6.75(♂)$; $F = 11.56(♀)$). In all years up to 1983, the mean length-at-age of males and females was smaller for fish recaptured in the inside waters compared to those recaptured in outside waters (Table 1). For males, length differences were significant (ANOVA, $P \leqslant 0.05$; $F = 7.71$). For females, when sample sizes were small, no significant difference was detectable. After 1983, the numbers of fish recaptured were small, making it difficult to compare growth differences between fish recaptured in inside and outside waters.

Tagged fish recaptured from outside waters were significantly smaller than untagged fish from the same 1977 year-class (Table 2) (ANOVA, $P \leqslant 0.05$; $F = 22.85(♂)$; $F = 96.89(♀)$) for both the 1979 and 1980 releases. The mean lengths of the tagged fish recaptured from the 1979 releases were slightly, but not significantly, larger than those from the 1980 release.

Differences in length between tagged and untagged fish from the 1977 year-class occurred in the first year of recapture and by age seven untagged females were over 8 cm longer than tagged females (Table 2). These length differences continued to increase with age. Similarly, by age four, males showed a significant difference in length between tagged and untagged fish. By age seven, untagged males were 6.5 cm larger than tagged males.

TABLE 3. Mean lengths by state of maturity of juvenile sablefish (1977 year-class) recovered in outside waters by depth.

Release year	Maturity state	1979 M	1979 F	1980 M	1980 F	1981 M	1981 F	1982 M	1982 F	1983 M	1983 F	1984 M	1984 F	1985 M	1985 F	1986 M	1986 F	1987 M	1987 F
		Outside Shallow (<200 m)																	
1979	Immat.	—	—	46.7(1)	45.6(1)	49.9(2)	55.8(2)	—	50.1(2)	57.8(3)	62.9(2)	54.4(1)	—	—	—	52.9(1)	—	—	—
	Mature	—	—	—	—	51.2(2)	—	—	—	—	77.0(1)	54.7(2)	61.8(1)	58.5(1)	50.2(3)	56.4(3)	75.0(1)	60.8(1)	74.1(4)
1980	Immat.	—	—	—	—	—	55.0(1)	—	53.0(1)	—	54.5(2)	54.6(1)	51.9(1)	52.7(1)	59.0(1)	—	—	—	—
	Mature	—	—	—	—	50.0(1)	45.4(1)	—		56.2(2)	60.0(1)	50.1(1)	59.2(1)	55.0(1)	59.3(2)	59.4(3)	69.6(1)	—	70.4(1)
		Outside Deep (≥200 m)																	
1979	Immat.	—	—	47.6(1)	53.8(1)	49.9(6)	51.8(6)	51.1(3)	50.4(5)	55.3(4)	60.0(1)	51.1(1)	63.1(1)	—	—	—	—	—	—
	Mature	—	—	51.0(1)	45.7(2)	66.2(1)	55.9(4)	54.7(1)	—	56.7(1)	58.0(1)	58.0(2)	61.9(3)	—	—	—	76.8(1)	—	—
1980	Immat.	—	—	—	—	50.9(2)	51.3(2)	54.1(1)	50.6(3)	51.7(2)	62.5(2)	51.0(1)	—	—	—	—	—	—	—
	Mature	—	—	—	—	—	—	—	—	49.7(2)	59.0(1)	—	62.8(2)	—	60.6(1)	—	—	—	—

TABLE 4. Discards of juvenile sablefish (tonnes) in the inside waters (Hecate Strait — Queen Charlotte Sound) as reported from the Canadian trawl fishery (McFarlane and Beamish 1983).

Year	Tonnes
1977	9
1978	286
1979	1661
1980	1111
1981	266
1982	36
1983	7
1984	30

TABLE 5. Release and recapture percentages of juvenile sablefish tagged and released in 1979 and recaptured in inside and outside waters 1980–82.

Length groups (cm)	Release %	Release n	Recapture Inside %	Recapture Inside n	Recapture Outside %	Recapture Outside n
40–44	52	(12 107)	54	(75)	37.7	(61)
45–49	46	(10 768)	40	(55)	48.8	(79)
50+	2	(616)	6	(8)	13.6	(22)

TABLE 6. Mean age and length at 50% maturity of sablefish by year by sex for fish captured in the commercial fishery, (A) untagged fish caught in outside waters, and (B) tagged recaptured 1977 year-class as calculated using probit analysis.

	Males Age	Males Length (cm)	Females Age	Females Length (cm)
(A) Commercial fishery				
1982	3.8	53.6	3.8	52.7
1983	4.9	53.9	5.0	51.7
1984	3.8	53.8	3.9	54.0
1982–84	4.2	53.8	4.7	52.7
(B) Recaptured tagged 1977 year-class				
Inside waters	6.3	53.1	6.1	60.0
Outside waters	5.4	52.1	6.2	59.1

Tagged fish recaptured from outside waters at depths shallower than 200 m were approximately the same length as tagged fish recaptured from deeper waters (Table 3). Some differences in mean length by depth occurred among years, but these differences were much smaller than the length differences between tagged and untagged fish.

Movement to Outside Waters

The 1979 releases were recaptured in the inside area from 1980 to 1982. Incidental catches of juveniles in the trawl fishery indicated that the 1977 year-class moved from inside waters to outside waters by 1982 (Table 4). Discards of juvenile sablefish from these fisheries increased to a maximum level of 1 661 tin 1979. By 1981, discards declined to 266 t. After 1981, discards were approximately equal to levels observed prior to 1977. After 1982, only occasional catches of juveniles were made in the trawl fishery in inside waters.

Percentage Recapture of Smallest Length Groups

Sablefish tagged in 1979 ranged in size from 27–54 cm, 95% of these fish were 40–49 cm. In inside waters, the frequency of recaptures in size groups 40–44 cm, 45–49 cm, and 50+ cm was 54, 40, and 6%, respectively, for the period 1980–82. The percentage of recaptures in the two smaller size groups was not significantly different (x^2 $P \geqslant 0.05$) from the percentage released in each of these size groups in 1979 and 1980 (Table 5). However, the recapture percentage was higher than the release percentage for the few fish larger than 50 cm. In contrast, when fish were recaptured from outside waters, the total recapture percentage in the smallest size group (40–44 cm) was significantly smaller than the percentage released (*t*-test, $P \leqslant 0.05$). There was no difference between the release percentage for inside waters and the recapture percentage for outside waters for the 44–49 cm size group. This indicates that the recapture percentage for fish from the smallest size category was smaller throughout the study for fish recaptured from outside waters when compared with the recapture percentage for fish recaptured from inside waters.

Size and Age at Maturity

In the inside waters, 50% of the tagged males were mature at a mean length of 53.1 cm and tagged females at 60.0 cm (Table 6). Males first matured at age three and a length of 45.9 cm; females at age three and a length of 51.1 cm (Table 7). Age at 50% maturity for both sexes was age six (Table 6). In the outside waters, recaptured fish first matured at age three and a length of 51.0 cm for males and 45.7 cm for females (Table 8). Fifty percent of the males were mature at age five and a length of 52.1 cm, and females at age six and a length of 59.1 cm (Table 6).

In comparison, the mean length at 50% maturity of untagged commercially caught sablefish from the 1977 year-class was 53.8 cm for males and 52.7 cm for females. The age at 50% maturity for these untagged fish was 4+ yr, 1 yr younger than the tagged, recaptured males and 1.5 yr younger than the tagged, recaptured females from outside waters.

In 1986, at age nine, two of 13 recaptured fish in inside waters were still immature. Mature males and females were first captured in 1980 (age 3 yr), but only 6% of the recaptures were mature. Although the percentage of fish that were mature increased from 1980 to 1987, and few fish were recaptured in the inside waters after 1981, many recaptures in the inside waters were immature. In contrast, the majority (81%) of recaptures in the outside waters were mature after 1983 (age 6 yr). A few fish recaptured in the outside waters remained immature as of December 31, 1985 (age 8 yr).

Discussion

Decreased Growth of Tagged Fish

When the 1977 year-class recruited to the commercial trap fishery, it was possible to obtain samples of "untagged" fish. In all years after 1980, the mean length of untagged fish was significantly larger than the tagged fish. This difference in length between tagged and untagged fish was not the result of size related movements. Juveniles moved to outside waters by 1981 and were recruited to the fishery. Once recruited to the fishery, there was no evidence of size segregation by depth. This means the difference in length could have resulted from

TABLE 7. Mean lengths by state of maturity for juvenile sablefish (1977 year-class) recovered in inside waters, 1979–87 (total number of fish in parenthesis).

Release year	Maturity state	Recovery year 1979 M	1979 F	1980 M	1980 F	1981 M	1981 F	1982 M	1982 F	1983 M	1983 F	1984 M	1984 F	1985 M	1985 F	1986 M	1986 F	1987 M	1987 F
1979	Immat.	45.7(3)	48.0(4)	46.0(33)	46.7(48)	49.5(3)	52.5(8)	52.6(4)	52.6(7)	50.5(2)	55.1(3)	53.5(3)	—	—	—	—	—	—	—
	Mature	—	—	45.9(2)	51.1(3)	49.9(3)	—	59.0(1)	68.4(3)	—	60.8(2)	57.8(1)	—	—	—	—	61.3(2)	70.7(3)	—
1980	Immat.	—	—	—	51.5(6)	49.0(7)	51.6(4)	47.6(3)	50.1(2)	53.9(4)	56.4(1)	52.1(2)	—	—	—	—	—	—	—
	Mature	—	—	—	—	50.2(9)	55.8(11)	54.7(6)	50.0(1)	51.0(1)	—	49.8(1)	62.4(2)	54.2(1)	—	—	—	65.4(1)	—

TABLE 8. Mean lengths by state of maturity for recaptured juvenile sablefish (1977 year-class) recovered in outside waters, 1979–87 (total number of fish in parenthesis).

Release year	Maturity state	Recovery year 1979 M	1979 F	1980 M	1980 F	1981 M	1981 F	1982 M	1982 F	1983 M	1983 F	1984 M	1984 F	1985 M	1985 F	1986 M	1986 F	1987 M	1987 F
1979	Immat.	—	—	47.1(2)	49.7(2)	49.8(8)	52.8(8)	51.1(3)	50.3(7)	56.4(7)	61.9(3)	52.8(2)	63.1(1)	—	—	52.9(1)	—	—	—
	Mature	—	—	51.0(1)	45.7(2)	56.2(3)	55.9(4)	54.7(1)	—	56.7(1)	67.5(2)	56.3(4)	61.9(4)	58.5(1)	50.2(3)	59.5(4)	75.9(2)	60.8(1)	74.1(4)
1980	Immat.	—	—	—	—	50.9(2)	52.5(3)	54.1(1)	51.2(4)	51.8(2)	58.5(4)	52.8(2)	51.9(1)	52.7(1)	59.0(1)	—	—	—	—
	Mature	—	—	—	—	50.0(1)	—	45.4(1)	—	52.9(4)	59.5(2)	50.1(1)	61.6(3)	55.0(1)	59.7(3)	59.4(3)	69.6(1)	—	70.4(1)

TABLE 9. Frequency distribution (no. of fish) of estimated ages of recaptured (known age) fish.

Recapture year	Know age (yr)	Estimated age 3	4	5	6	7	8	9	10	(*n*)
1980	3 yr	10	2							(12)
1981	4 yr	1	12	2						(15)
1982	5 yr		2	13	5					(20)
1983	6 yr			1	7	1				(9)
1984	7 yr			1	4	16	7			(28)
1985	8 yr					3	6	2	1	(12)

untagged fish being aged incorrectly, or from slower growth of the tagged fish.

If untagged fish were underaged, growth would be overestimated, creating the impression that the tagged fish were slower growing. The ages estimated from known-aged fish (Table 9) indicated that there was ageing error and that the amount of error increased as the fish became older. However, there was little or no indication of a bias towards older or younger ages within this age range, indicating that the mean ages were correct and the average sizes at age were representative of the population. Thus, the only explanation is that the tag or the tagging operation reduced growth.

Increased Mortality of Smaller Tagged Fish

In another study (Saunders et al. 1990), it was shown that there was a significant relationship between size at tagging and the rate of recapture of tagged fish. For each increasing 5 cm interval, there was an increased percentage of fish recaptured. The differences in recapture percentages between the smallest and longest length categories was up to 25%. It is generally accepted that smaller fish have a higher mortality rate, but it was not anticipated that there would be such a strong, consistent, and incremental relationship between release size and recaptured percentage.

In this study, in the inside waters, we did not find a significant difference in the recovery rates of fish released at lengths of 40–44 cm and 45–50 cm. For these two smaller length groups, the percent recaptured in the inside waters was directly proportionate to the percent released. However, in the outside waters, there was a reduced recapture percentage of the smallest size group. The Saunders et al. (1990) study also showed that there was a reduced recapture percentage for the smallest tagged fish. This indicates there was a higher mortality rate for the smaller tagged fish. Our study showed that tagging reduces growth, that is, tagging results in an increased percentage of smaller fish. Tagged fish, therefore, will have a higher mortality rate. This also indicates that estimates of mortality from tagged fish overestimate the true rate of mortality. Our study showed that this increased mortality occurred when the tagged fish moved to the outside waters. Because the mortality occurred after the fish moved offshore, away from the nursery area, we believe this increased mortality results from predation.

Length and Age at 50% Maturity

Changes in size at sexual maturity can occur for genetic reasons (Alm 1959) or from a density-dependent growth and maturation relationship. Before the reasons for such changes can be studied, it is important that the estimate of size and age at maturity be determined correctly. The tagged females matured later, over a broader range of lengths, and at a larger length at 50% maturity than untagged fish of the same year-class. Assuming that the maturation process is not affected physiologically by the presence of the tag, the study indicates that size is more important than age in determining when sablefish first mature. Because tagged females were larger at 50% maturity than untagged females, the rate of growth is probably more important than absolute size in determining the onset of maturity. It is possible that the "surplus" energy needed for reproductive growth (Ware 1980) was not available until females were larger than average. Because fish in this study were of "known" age, it was possible to confirm that some fish were immature for as long as 10 yr.

Population Response to Reduced Growth

As mentioned in our introduction, there is considerable interest in the changes in age and size at maturity that occur in relation to changes in growth. Fishing strategies can be affected if exploitation or the ocean environment alter size and age at maturity. In our study, we unintentionally slowed the rate of growth. By slowing the rate of growth we demonstrated experimentally that the population response to reduced growth would be higher juvenile mortality, delayed age at 50% maturity, but increased mean length at 50% maturity. If the reduced growth results from a chronic, physiological problem associated with either the type of tag, and/or the tagging procedure, then the changes may not be directly related to growth. In an earlier report (Beamish and McFarlane 1988), we showed that adult sablefish tagged in the same manner were recaptured over 9 yr. The recapture rates were some of the highest reported and were close to the expected recapture rates that assumed a 0.05% initial tagging mortality, 0.1% natural mortality rate, 0.1% fishing mortality rate, and no long-term stress-related mortality. Thus, we suggest that growth changes were responsible for the increased age and length at 50% maturity and not the result of a chronic physiological stress.

Conclusion

In this study we observed that the placement of a tag in a fish altered its biology relative to untagged fish of the same year-class. Growth was slower resulting in increased mortality and the mean age at 50% maturity was greater compared to untagged fish. To our knowledge, this is the first comprehensive marine study to examine the effect of this type of tag on these biological parameters. If the results apply to other species, and other types of tags, as demonstrated by Saunders and Allen (1967), there could be errors in the estimation of biological parameters that are obtained from tagging studies. A number of authors have developed methods to analyze tagging results to obtain life history parameter estimates for a population. Our results indicate that before these procedures are used, it should be determined if the tagging operation is affecting the parameters that are estimated. We understand that it is difficult to carry out similar studies, but if a tagging process has as much effect as was observed in our study, investigators should be cautious about using estimates of biological parameters from tagging studies to represent the biology of the untagged population.

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References

ALM, G. 1959. Connection between maturity, size and age in fishes. Inst. Freshwater Res. Drottningholm. 40: 4–145.

BEAMISH, R. J., AND D. E. CHILTON. 1982. Preliminary evaluation of a method to determine the age of sablefish (*Anoplopoma fimbria*). Can. J. Fish. Aquat. Sci. 39: 277–287.

BEAMISH, R. J., C. HOULE, C. WOOD, AND R. SCARSBROOK. 1979. A summary of sablefish tagging and exploratory tagging studies conducted during 1978 by the Pacific Biological Station. Can. Data Rep. Fish. Aquat. Sci. 162: 113 p.

BEAMISH, R. J., AND G. A. MCFARLANE. 1983. The forgotten requirement for age validation in fisheries biology. Trans. Am. Fish. Soc. 112: 735–743.

1988. Resident and dispersal behavior of sablefish in the slope waters off Canada's west coast. Can. J. Fish. Aquat. Sci. 45: 152–164.

FOUCHER, R. P., AND D. FOURNIER. 1982. Derivation of Pacific cod age composition using length-frequency analysis. N. Am. J. Fish. Manage. 2: 276–284.

MASON, J. C., R. J. BEAMISH, AND G. A. MCFARLANE. 1983. Sexual maturity, fecundity, spawning, and early life history of sablefish (*Anoploma fimbria*) off the Pacific coast of Canada. Can. J. Fish. Aquat. Sci. 40(12): 2126–2134.

MCFARLANE, G. A., AND R. J. BEAMISH. 1983. Biology of adult sablefish (*Anoplopoma fimbria*) in waters off western Canada. p. 59–80. *In* Proceedings of the Second Lowell Wakefield Fisheries Symposium, Anchorage, AK. Alaska Sea Grant Report 83-8.

PARKS, N. B., AND F. R. SHAW. 1983. Changes in relative abundance and size composition of sablefish in coastal waters of Washington and Oregon, 1979–81 and California, 1980–82. p. 273–284. *In* Proceedings of the Second Lowell Wakefield Fisheries Symposium, Anchorage, AK. Alaska Sea Grant Report 83-8.

SAUNDERS, R. L., AND K. R. ALLEN. 1967. Effects of tagging and of fin-clipping on the survival and growth of Atlantic salmon between smolt and adult stages. J. Fish. Res. Board Can. 24: 2595–2611.

SAUNDERS, M., G. A. MCFARLANE, AND R. J. BEAMISH. 1990. Factors that affect the recovery rates of sablefish tagged off the west coast of Canada. Am. Fish. Soc. Proc. Int. Symp. and Educational Workshop on Fish-Marking Techniques, Seattle, WA, June 27–July 1, 1988. (In press)

SINCLAIR, M., A. SINCLAIR, AND T. D. ILES. 1982. Growth and maturation of southwest Nova Scotia Atlantic herring (*Clupea harengus harengus*). Can. J. Fish. Aquat. Sci. 39: 288–295.

Ware, D. M. 1980. Bioenergetics of stock and recruitment. Can. J. Fish. Aquat. Sci. 37: 1012–1024.

WELCH, D., AND R. FOUCHER. 1988. A maximum likelihood methodology for estimating length-at-maturity with application to Pacific cod (*Gadus macrocephalus*) population dynamics. Can. J. Fish. Aquat. Sci. 45: 333–343.

Is Precipitation of Aluminum Fast Enough to Explain Aluminum Deposition on Fish Gills?

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Playle, R. C., and C. M. Wood. 1990. Is precipitation of aluminum fast enough to explain aluminum deposition on fish gills? Can. J. Fish. Aquat. Sci. 47: 1558–1561.

A peristaltic pump was used to mix environmentally realistic concentrations of Al in soft water (14–15°C) with 30 μM base (NH_4OH) to simulate the alkalinization which occurs when acidic soft water passes over fish gills. Solutions were filtered under vacuum through 0.2 μm prerinsed filters using a polycarbonate filter apparatus. If final solution pH was $\leq$5.3, only about 10% of total Al (159 μg·L^{-1}) was filtered from solution. If solution pH was raised from pH 5.3 to pH 5.7 with base, about 35% of Al was removed from solution, 1–4 s after the pH was raised. Similar results were obtained for 335 μg·L^{-1} Al solutions. Production of additional Al retained by filters was fast enough to show that Al accumulation on fish gills may be partly due to polymerisation and precipitation of Al, as Al solubility is reduced in the more alkaline gill micro-environment.

On a utilisé une pompe péristaltique pour incorporer du Al dilué dans de l'eau douce (14–15°C) avec 30 μM de base (NH_4OH) afin de reproduire l'alcalinisation qui se produit quand des eaux douces acides traversent les ouies des poissons. On a filtré les solutions sous vide avec des filtres pré-rinsés de 0,2 μm à l'aide d'un appareil de filtrage au polycarbonate. Si le pH de la solution finale était inférieur à 5,3, moins de 10 % de la concentration totale de Al (159 μg·L^{-1}) était filtrée de la solution. Si, par ajout de base, le pH passait de 5,3 à 5,7, environ 35 % du Al était enlevé de la solution de une à quatre secondes après l'augmentation du pH. On a obtenu des résultats semblables avec des solutions de 335 μg·L^{-1}. La production de Al supplémentaire retenu par le filtre était assez rapide pour révéler que l'accumulation de Al dans les ouies des poissons peut être en partie le résultat de la polymérisation et de la précipitation du Al étant donné que sa solubilité est diminuée dans le milieu plus alcalin des ouies.

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Solubility of Al is dependent on water pH, falling exponentially as pH rises from pH 4, until minimum solubility is reached at about pH 5.8 (eg. Roberson and Hem 1969). Recently, we showed that the pH of acidic soft water increases as it passes over rainbow trout gills, because the fish's metabolism releases approximately 30 μM of base (ammonia plus unidentified titratable alkalinity; Playle and Wood 1989a). The extent of the pH rise depends on inspired water pH and the acidifying influence of CO_2 released at the gills. If inspired soft water contains 100–200 μg·L^{-1} Al, the pH increase at the gills could theoretically cause a loss of Al solubility at inspired pHs 4.8–5.8 (cf. figure 5 of Playle and Wood 1989b). Reduced Al solubility in the gill micro-environment would result in Al deposition on the gills. Accumulation of Al on fish gills appears to be the cause of disturbances in ionoregulation and respiratory gas transfer which may kill the fish (eg. Neville 1985; Neville and Campbell 1988; and others listed in Playle et al. 1989).

However, it was not known if Al could precipitate from solution fast enough to account for Al deposition on fish gills. In the past, researchers working with Al chemistry have usually been interested in geological time scales rather than in biological time scales, and have often measured Al precipitation from concentrated Al solutions over hours to days (eg. May et al. 1979; Parthasarathy and Buffle 1985). The fastest reported time for Al precipitation is 1 min, for 540 or 1080 μg·L^{-1} Al solutions at 2–20°C (Tipping et al. 1988). As a result, we measured Al precipitation over a time scale approximating water residence time at fish gills, in acidic soft water of low Al concentration, with an alkalinization of the water similar to that previously measured at fish gills.

Methods

Soft water was produced by passing dechlorinated Hamilton city tapwater through a deionising resin cannister (J.W. Anderson Co. Ltd., Dundas, Ont.) or through a reverse osmosis unit (Culligan MP1000), and adding appropriate amounts of analytical grade NaCl and $CaCl_2$ (BDH, Toronto, Ont.). Water composition was: Ca^{2+} ~40 μeq·L^{-1}, Na^+ ~50 μeq·L^{-1}, Cl^- ~90 μeq·L^{-1}, titratable alkalinity to pH 4.0 ~130 μeq·L^{-1}, pH ~6.5. Aluminum solutions were made in this water by adding a concentrated stock solution of $AlCl_3 \cdot 6H_2O$ (0.39 g·L^{-1}, pH ~4, Sigma).

Preliminary experiments on Al precipitation rates used a Hach 2100A turbidity meter, a Pye Unicam PU 8600 spectrophotometer, and a glass 300 mL filtering funnel (Millipore) with 0.2 or 0.45 μm membrane filters (Millipore). Aluminum concentrations from these experiments were measured using the pyrocatechol violet method (Dougan and Wilson 1974).

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Final experiments used a peristaltic pump (Gilson Minipuls 2) to mix the test Al solution (7.2 mL·min^{-1}) with deionised water or 300 µM NH_4OH (0.7 mL·min^{-1} for both). The dilution resulted in about 30 µM base addition. Deionised water addition was the control. All solutions were kept at 14–15°C. Initial pH of the Al solution was adjusted with dilute H_2SO_4. Final solution pHs (after mixing with base or water, but before being filtered) were measured in 8 mL polyethylene vials (stirred) using a Radiometer PHM82 meter and Radiometer GK2401C combination electrode. One minute was allowed for electrode stabilisation.

The Al solution and base (experimental) or Al and water (control) met at a polyethylene "T", then flowed along either 2 or 16 cm of polyethylene tubing, which took 0.8 or 3.2 s aftermixing, respectively. The solution then dripped onto a 0.2 µm Nuclepore filter under vacuum (700 mm Hg) and was collected in an 8 mL polyethylene vial suspended beneath the filtering apparatus. Total delay between mixing and arrival of a drop in the vial after filtration was estimated to be about 1.4 or 3.8 s. The filter funnel assembly was plastic (polycarbonate; Nuclepore), with a rubber O-ring and silicone gasket. The funnel was disassembled completely between each sample run, rinsed in 5% HCl, distilled water, then with deionised water. The re-assembled filtering apparatus and fresh 0.2 µm filter were rinsed with two 100 mL volumes of 5% HCl under vacuum, then with two 100 mL volumes of soft water acidified to pH~4.3 with dilute H_2SO_4, and finally rinsed for 2 min with the experimental or control solution dripping onto the filter, again under vacuum. This protocol eliminated Al contamination between samples. The third or fourth minute of filtered Al solution was the sample collected for analysis; filters did not clog in this time.

Samples (7 mL) from the final set of experiments were acidified with 20 µL concentrated HCl, refrigerated, then analysed without dilution for total Al using a Varian AA-1275 atomic absorption spectrophotometer with GTA-95 graphite tube atomizer. Ten µL of sample were analysed against standards at 309.3 nm. Operating parameters were as follows: 5 s at 80°C, 35 s at 95°, 10 s at 110°, 12 s at 1200°, and 2.7 s at 2 500°C, N_2 gas. Unpaired Student's *t*-tests were used for statistical analysis of Al data.

Results

Initial experiments demonstrated that colloidal Al formed quickly in concentrated solutions brought to near neutral pH. For example, a 44 mg·L^{-1} Al solution brought from pH 4.0 to pH 6.4 with 1 N KOH (at room temperature) turned cloudy within 3 min. On the turbidity meter, turbidity increased by 1.2 FTU units 25 s after base addition. Absorbance (291 nm) on the spectrophotometer increased by 0.006 units about 15 s after base addition. However, it became clear that these methods were not sensitive enough or quick enough to measure fast Al precipitation in dilute solutions, so filtering was tried.

Filtering a concentrated Al solution (44 mg·L^{-1}) 10 min after partial neutralisation (pH 4.0 brought to pH 5.8 with 1 N KOH) removed 90–100% of total Al, whereas filtering the same solution at pH 4.0 removed only about 4% of the Al. A glass filtering apparatus and 0.45 µm membrane filter were used. With a more reasonable concentration of Al (505 µg·L^{-1} at pH 5.3, 15°C), filtration through a 0.45 or 0.2 µm membrane removed about 25 and 36% of total Al, respectively. About 93% of the Al was removed when the same solution was brought to pH 6.4 and filtered within 3–5 min. However, for lower concentrations of Al, it became apparent that Al was being removed from solution by the glass filtering apparatus and glass porous filter support, as well as by the filters, so a plastic filtering unit was tried.

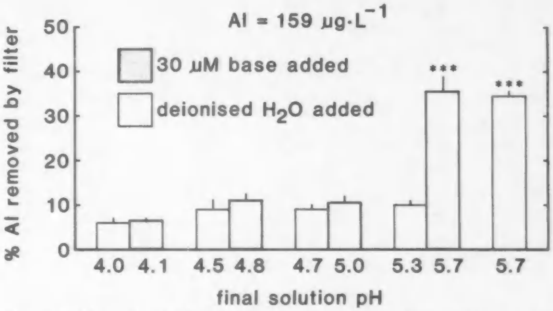

FIG. 1. Effects of solution pH on the amount of Al filtered from a 159 µg·L^{-1} Al soft water solution, expressed as a percentage of total Al. 14–15°C. Clear bars: percent of total Al removed from solution when filtered through a 0.2 µm filter 1.4 or 3.8 s after mixing with deionised water (control). Combined data from 1.4 and 3.8 s tests. Adjacent dark bars: percent of total Al removed when solution pH was raised from control values by mixing with base (30 µM NH_4OH). Combined data for 1.4 or 3.8 s tests. About 35% of total Al was removed by filtration if final solution pH was 5.7; only about 10% was removed if pH was ≤5.3. *** = $P<0.001$ (*t*-test), vs. pH 5.3 (control). For solution pHs ≤5.0, $n=6$; for pH 5.3, $n=16$; for pH 5.7 (dark bar) $n=18$; and for pH 5.7 (clear bar) $n=10$. Error bars: 1 SEM.

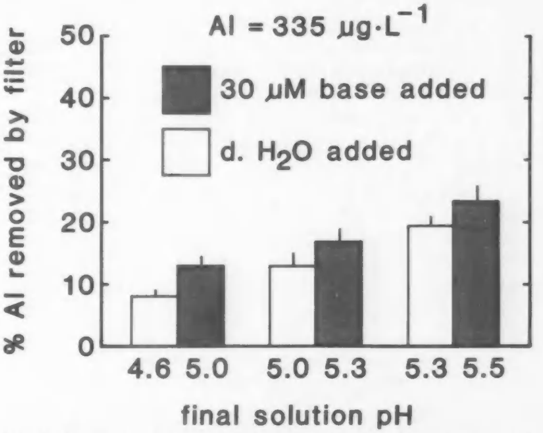

FIG. 2. Effect of solution pH on the amount of Al filtered from a 335 µg·L^{-1} Al soft water solution, expressed as a percentage of total Al. Delay after base or water addition to the solution and filtering was 1.4 s. Other details as in legend of Fig. 1. The trend was more Al removed as solution pH increased. For all bars, $n=3$.

Final experiments used a plastic filtering apparatus, and a peristaltic pump to reduce the time between base addition and filtering. For a 159 ± 2 µg·L^{-1} Al solution in soft water, ~10% of total Al was removed by filtering if the final pH of the solution was ≤5.3 (Fig. 1). If final solution pH was 5.7, or was raised to that pH by 30 µM NH_4OH addition, the amount of Al removed by filtering increased to about 35% of total Al. This effect was seen just 1.4 s after the pH rise induced by NH_4OH addition. There was no indication that the amount of Al removed from solution depended on the delay between base and Al solution mixing and filtration, therefore results from the 1.4 and 3.8 s delay tests have been pooled in Fig. 1.

To see if the same pattern of Al removal occurred from a more concentrated solution of Al at comparable pHs, experiments with a 335 ± 5 $\mu g{\cdot}L^{-1}$ Al solution were run. The pattern of increased Al removal from solution after base was added was still evident (Fig. 2), and, if the amounts removed are expressed as concentrations, more A1 was removed from the more concentrated Al solution at a given pH. However, the trend of greater Al removal at higher pH was less pronounced, partly because of the small number of replicates run, but also because of smaller increases in pH as 30 μM NH_4OH was added.

Discussion

The exact mechanisms leading to the increases in Al retained by filters at pH>5.3 (Fig. 1) are not known. As pH increases and Al solubility is reduced, monomers of Al may polymerise and form filterable complexes, or Al monomers may adsorb to the filter surface, polymerise, and form a precipitate (Dentel and Gossett 1988). These processes presumably occur at fish gills, as suggested by Baker and Schofield (1982). The increase in pH of ~0.4 units is about the difference between inspired and expired water pH seen in rainbow trout in similar soft water (Playle and Wood 1989a,b), and is large enough to theoretically result in Al precipitation if initial Al concentration is >100 $\mu g{\cdot}L^{-1}$ (Playle and Wood 1989b).

Residence time of water at the gills is 0.4–2 s (Randall 1970), and may be longer because of unstirred boundary layers, so the time scale of the filtration experiments was appropriate. Background removal of Al by filtration was about 10% of total Al, similar to the loss of Al from a standard solution filtered through rinsed, 0.4 μm filters (Campbell et al. 1983). Background removal of Al probably represents adsorption of positively charged Al species to negative charges known to exist on membrane filters (eg. Bisio et al. 1980; Johnson et al. 1989). Similarly, negative charges are assumed to exist on branchial surfaces, because of carboxyl groups on mucus (Satchell 1984). The filtration method used here, although crude, was able to simulate chemical conditions currently thought to exist near fish gills, in magnitidue of pH change, time scale, and even surface charge.

The solubility of Al is lower, and reaction rates faster, in water of low ionic strength because activity coefficients are greater at lower ionic strength (eg. Bache 1986). The low ionic strength of the soft water used ($\sim 10^{-4}$ M) would be expected to maximise Al precipitation rates. Aluminum filtered from solution was about 10% of total Al at pH ≤5.3, increasing to 35% whether the solution was brought to pH 5.7 just seconds before filtration, or was prepared at pH 5.7 and filtered minutes later (Fig. 1). In contrast, Chappel and Birchall (1988) reported only about 20% retention of Al at pH 5.7 compared to about 10% at pH 5.3 (20 h after solutions were prepared, 0.2 μm filters, ~3 $mg{\cdot}L^{-1}$ Al solution). These workers, however, used water of ionic strength about 10^{-2} M, which would have an Al activity coefficient about two–thirds that in the soft water used here (extended Dubye–Hückel equation, from Stumm and Morgan 1981). This could explain their apparently higher Al solubility even after 20 h.

The 14–15°C conditions used in our experiments would have slowed Al polymerisation and precipitation rates compared to rates at room temperature. However, Al precipitation as determined by filtration still occurred within seconds. Aluminum precipitation would be slowed further in very cold water (eg. 0–4°C), which would represent conditions encountered by fish during springmelt pulses of Al and acidity, but it is not known whether pH changes at the gills would be the same at these temperatures as at 15°. For example, fish not eating at cold temperatures might release more ammonia at the gills due to catabolism of endogenous protein, which would worsen Al precipitation. Alternatively, they might release less ammonia due to lowered metabolic rates. Clearly this question deserves more attention before chemical simulations of the gill micro-environment are done at lower temperatures.

As expected, more Al was filtered on an absolute basis from the 335 $\mu g{\cdot}L^{-1}$ Al solution at a given pH compared to the 159 $\mu g{\cdot}L^{-1}$ Al solution (Fig. 2). 335 $\mu g{\cdot}L^{-1}$ is close to saturation at pH 5.0 (eg. Roberson and Hem 1969). Certainly at pH ≥5.3 the Al solution is well above saturation, as indicated by the 17–24% removal of Al by filtration (Fig. 2). For the 335 $\mu g{\cdot}L^{-1}$ Al solution at pH 5.3, the addition of 30 μM base raised its pH to 5.5, whereas for the 159 $\mu g{\cdot}L^{-1}$ Al solution, pH increased to 5.7. This difference was likely due to the extra pH buffering of more Al in solution, because the formation of Al-hydroxides consumes base (eg. calculations in Playle and Wood 1989b).

Reduced Al solubility in the gill micro-environment can explain higher gill Al accumulation at inspired pH 5–6, but does not explain Al accumulation in more acidic water (eg. Neville 1985; Playle and Wood 1989b). Below pH 5, adsorption and complexation of positively charged Al species onto branchial surfaces and mucus better explain the smaller amount of Al accumulating on fish gills. The relative contribution of polymerisation, precipitation, adsorption, and complexation to gill Al deposition would be influenced by factors such as pH of the gill micro-environment, Al concentration, solution ionic strength and buffer capacity, negative charges on mucus and gill surfaces, the amount of mucus produced at the gills, and gill surface area.

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References

BACHE, B. W. 1986. Aluminium mobilization in soils and waters. J. Geol. Soc. (Lond.) 143: 699–706.

BAKER, J. P., AND C. L. SCHOFIELD. 1982. Aluminum toxicity to fish in acidic waters. Water Air Soil Pollut. 18: 289–309.

BISIO, P. D., J. G. CARTLEDGE, W. H. KEESOM, AND C. J. RADKE. 1980. Molecular orientation of aqueous surfactants on a hydrophobic solid. J. Colloid Interface Sci. 78: 225–234.

CAMPBELL, P. G. C., M. BISSON, R. BOUGIE, A. TESSIER, AND J.-P. VILLENEUVE. 1983. Speciation of aluminum in acidic freshwaters. Anal. Chem. 55: 2246–2252.

CHAPPELL, J. S., AND J. D. BIRCHALL. 1988. Aspects of the interaction of silicic acid with aluminium in dilute solution and its biological significance. Inorg. Chim. Acta 153: 1–4.

DENTEL, S. K., AND J. M. GOSSETT. 1988. Mechanisms of coagulation with aluminum salts. J. Am. Water Works Assoc. 80(4): 187–198.

DOUGAN, W. K., AND WILSON, A. L. 1974. The absorptiometric determination of aluminium in water. A comparison of some chromogenic reagents and development of an improved method. Analyst (London) 99: 413–430.

JOHNSON, K. A., G. B. WESTERMANN–CLARK, AND D. O. SHAH. 1989. Diffusion of charged micelles through charged microporous membranes. Langmuir 5: 932–938.

MAY, H. M., P. A. HELMKE, AND M. L. JACKSON. 1979. Gibbsite solubility and thermodynamic properties of hydroxy–aluminum ions in aqueous solution at 25°C. Geochim. Cosmochim. Acta 43: 861–868.

NEVILLE, C. M. 1985. Physiological response of juvenile rainbow trout, *Salmo gairdneri,* to acid and aluminum — prediction of field responses from laboratory data. Can. J. Fish. Aquat. Sci. 42: 2004–2019.

NEVILLE, C. M., AND CAMPBELL, P. G. C. 1988. Possible mechanisms of aluminum toxicity in a dilute, acidic environment to fingerlings and older life stages of salmonids. Water Air Soil Pollut. 42: 311–327.

PARTHASARATHY, N., AND J. BUFFLE. 1985. Study of polymeric aluminium (III) hydroxide solutions for application in waste water treatment. Properties of the polymer and optimal conditions of preparation. Water Res. 19: 25–36.

PLAYLE, R. C., AND C. M. WOOD. 1989a. Water chemistry changes in the gill micro-environment of rainbow trout: experimental observations and theory. J. Comp. Physiol. B. 159: 527–537.

1989b. Water pH and aluminum chemistry in the gill micro-environment of rainbow trout during acid and aluminum exposures. J. Comp. Physiol. B. 159: 539–550.

PLAYLE, R. C., GOSS, G. G., AND C. M. WOOD. 1989. Physiological disturbances in rainbow trout (*Salmo gairdneri*) during acid and aluminum exposures in soft water of two calcium concentrations. Can. J. Zool. 67: 314–324.

RANDALL, D. J. 1970. Gas exchange in fish, p. 253–292. *In* W. S. Hoar and D. J. Randall [ed.] Fish Physiology Vol. IV. Academic Press, New York, NY.

ROBERSON, C. E., AND J. D. HEM. 1969. Solubility of aluminum in the presence of hydroxide, fluoride, and sulfate. U.S. Geol. Surv. Water Supply Pap. 1827c.

SATCHELL, G. H. 1984. Respiratory toxicology of fishes. *In* L. J. Weber [ed]. Aquatic toxicology. Raven Press, New York, NY.

STUMM, W., AND J. J. MORGAN. 1981. Aquatic chemistry. John Wiley and Sons, Toronto, Ont. 780 p.

TIPPING, E., C. WOOF, P. B. WALTERS, AND M. OHNSTAD. 1988. Conditions required for the precipitation of aluminium in acidic natural waters. Water Res. 22: 585–592.

Chromosomal and Mitochondrial DNA Characterization of a Population of Dwarf Cisco (*Coregonus artedii*) in Minnesota[1]

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Shields, B. A., K. S. Guise, and J. C. Underhill. 1990. Chromosomal and mitochondrial DNA characterization of a population of dwarf cisco (*Coregonus artedii*) in Minnesota. Can. J. Fish. Aquat. Sci. 47: 1562–1569.

This study compared the chromosome complement and mitochondrial DNA profile of a population of dwarf cisco in Ten Mile Lake, Cass County, Minnesota, to two populations of normal-sized *Coregonus artedii* in order to determine if the dwarf fish were conspecific with *C. artedii*. Chromosomal analysis revealed a diploid chromosome number ($2n = 80$) the same as reported for *C. artedii*. Mitochondrial DNA (mtDNA) analysis revealed unexpected levels of intrapopulational diversity in each study population, with the dwarf fish displaying the highest level of mtDNA diversity. Heteroplasmy, restriction site polymorphisms, and three size variants were documented. Each population was characterized by a unique profile of mtDNA clonal types, yet interpopulational comparisons revealed more similarities between the dwarf fish in Ten Mile Lake and the two normal populations than between the populations of normal-sized fish. Based on these data, the dwarf fish of Ten Mile Lake should be considered conspecific with *Coregonus artedii*.

La présente étude compare le complément chromosomique et le profil de l'ADN mitochondrial d'une population de cisco nain du lac Ten Mile, comté de Cass (Minnesota), à deux populations de *Coregonus artedii* de taille normale afin de déterminer si le poisson nain et *C. artedii* appartenaient à la même espèce. L'analyse chromosomique a révélé que le nombre de chromosome diploïde ($2n = 80$) était le même que celui relevé chez *C. artedii*. L'analyse de l'ADN mitochondrial (mtADN) a montré un taux inattendu de diversité entre les populations au sein de chacune des populations à l'étude, le taux le plus élevé de diversité du mtADN se trouvant chez les poissons nains. L'hétéroplasmie, les polymorphismes des sites de restriction, et trois variants de taille ont été étayés. Chaque population était caractérisée par un profil unique de types de clones de mtADN, quoique des comparaisons entre les populations aient révélé plus de similitudes entre le poisson nain du lac Ten Mile et les deux populations normales qu'entre les populations de poissons de taille normale. D'après ces données, les poissons nains du lac Ten Mile devraient être considérées comme des congénères de *Coregonus artedii*.

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Morphological plasticity and intraspecific variability of coregonine fishes have resulted in taxonomic confusion and management problems in Eurasia as well as North America (Steinmann 1951; Svaerdson 1970; Nikolsky and Reshetnikov 1970; Scott and Crossman 1973; Smith and Todd 1984). Many of these problems could be resolved by ascertaining the relative contributions of environment and genotype towards determining the characteristic morphotype of a given stock or population of *Coregonus*.

Attempts to resolve such questions at the organismal level have yielded equivocal results. Sympatric dwarf and normal populations of *Coregonus* sp. have been well-documented (Fenderson 1964; Lindsey et al. 1970; Mann and McCart 1981), indicating a possible genetic basis to the morphological variation observed in some stocks. However, studies on a variety of species of North American ciscoes (subgenus *Leucichthys*) reared in captivity by Todd et al. (1981) illustrate that incubation and rearing conditions may alter the morphology of these fishes significantly from their free-living native stocks. In addition, transplantation studies have documented that at least some populations of dwarf *Coregonus* possess potential for growth if they are transferred from their native lakes (Svaerdson 1970; J. C. Underhill, unpubl. data). Such studies indicate the strong influence of the environment in determining the overall phenotype of these fishes.

Since the instability of morphological traits renders them unreliable for discrimination between populations or species of this highly plastic species complex, efforts have focused on features which are less subject to environmentally influenced

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[1] Contribution No. 17418 of the Minnesota Agricultural Experiment Station based on research supported by the Station.

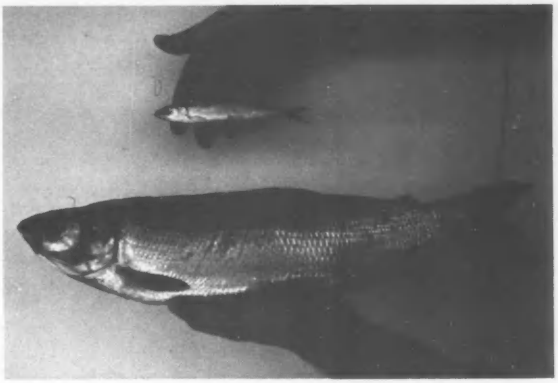

FIG. 1. Both ciscoes are mature females, collected on their spawning grounds in 1987. The dwarf female, an exceptionally large fish from Ten Mile Lake, weighs barely 18 g, including her entire egg burden. Since she was in her third year of life, this was probably her second and last spawning season. The normal-sized female from Six Mile Lake weighs over 800 g, was estimated to be in her eighth year of life, and may have survived to spawn a total of five or more times during a normal lifespan. A lack of pigmentation in the ventral fins, several morphometric measurements, and an atypical spawning season distinguish the dwarf fish from other ciscoes in north-central Minnesota.

alteration. Sola (1989) was able to discriminate stocks of European coregonines on the basis of chromosome number. Advances in molecular genetics have expanded the variety of techniques available to the fisheries manager (Hallerman and Beckman 1988; Kapuscinski and Philipp 1988). One method in particular, mitochondrial DNA (mtDNA) analysis, has been employed successfully to identify stocks of a variety of fish species (Gyllensten and Wilson 1987; Billington and Hebert 1988; Bentzen et al. 1988). Because mtDNA has a high natural mutation rate and evolves almost independently from nuclear DNA (Brown et al. 1979), it may document differences between recently diverged populations before other genetic changes are readily apparent (Birkey et al. 1983; Moritz et al. 1987). If mtDNA analysis identifies certain races or stocks of fish as genetically distinct, such information may be utilized for conservation and management programs designed to prevent the extirpation of such stocks.

The present study utilized chromosomal and mtDNA analyses in an attempt to resolve the genetic relatedness of a population of dwarf cisco to two populations of normal-sized *C. artedii* within the same watershed.

Materials and Methods

Collection of Specimens

The populations of *C. artedii* studied were from three lakes in Cass County, Minnesota. Meristic, morphometric, and life history data have already been examined for the population of dwarf cisco in their native environment and in three different transplant lakes (B. A. Shields and J. C. Underhill, unpubl. data). The dwarf cisco is characterized by an unusually small size at maturity (Fig. 1), a shift in the spawning season, and a shortened lifespan, all of which distinguish them from other populations of cisco in the same watershed (B. A. Shields and J. C. Underhill, unpubl. data). All three study lakes are of similar glacial age and origin (Zumberge 1952) and lie within the Upper Mississippi River drainage basin (Fig. 2).

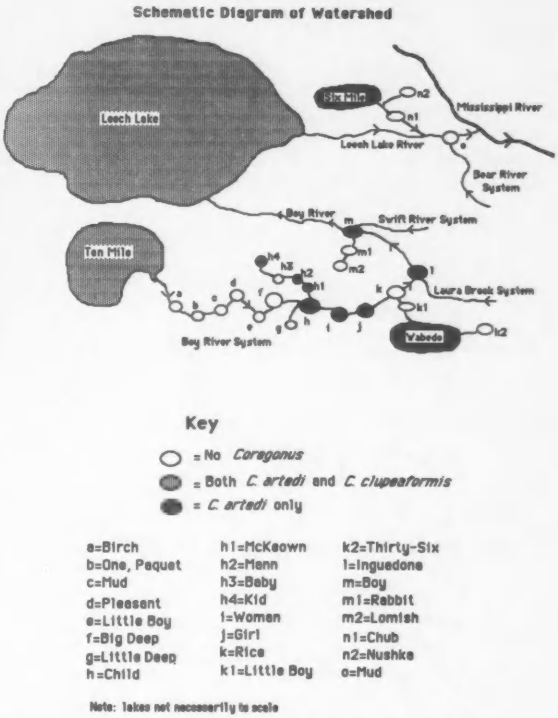
Schematic Diagram of Watershed
Leech Lake
Six Mile
n2
n1
Mississippi River
Leech Lake River
o
Bear River System
Boy River
m
Swift River System
Ten Mile
m1
m2
h4
h3
h2
h1
l
Laura Brook System
k
k1
a
b
c
d
e
f
g
h
i
j
Boy River System
Wabedo
k2
Key
= No Coregonus
= Both C. artedi and C. clupeaformis
= C. artedi only
a=Birch
b=One, Paquet
c=Mud
d=Pleasant
e=Little Boy
f=Big Deep
g=Little Deep
h=Child
h1=McKeown
h2=Mann
h3=Baby
h4=Kid
i=Woman
j=Girl
k=Rice
k1=Little Boy
k2=Thirty-Six
l=Inguedone
m=Boy
m1=Rabbit
m2=Lomish
n1=Chub
n2=Nushka
o=Mud
Note: lakes not necessarily to scale

FIG. 2. Schematic diagram of watershed depicting the locations of the three study populations. Ten Mile Lake, home of the dwarf cisco, is the origin of the Boy River, which interconnects many lakes in the area. Note the absence of *C. artedii* from many interconnecting lakes in the watershed.

TABLE 1. Lake characteristics, summarized from lake survey information obtained from the Minnesota Department of Natural Resources. Dwarf cisco are found in Ten Mile Lake, which is larger, deeper, and less mesotrophic than the other study lakes.

Lake characteristics	Ten Mile	Wabedo	Six Mile
Lake area (ha)	2085.8	479.8	521.5
Maximum depth (m)	63.4	28.9	20.7
Summer Secchi depth (m)	7.6	2.9	2.4
Summer kills of *Coregonus*	No	Yes	Yes
Sympatric *Coregonus* sp.	Yes	No	No[a]

[a] *Coregonus clupeaformis* reported in the lake by DNR and local residents prior to 1937, but apparently extirpated.

The population of dwarf cisco occurs in Ten Mile Lake (46°56′N, 94°35′W), which also supports a population of normal-sized lake whitefish (*C. clupeaformis*). The two populations of normal-sized cisco utilized for comparison are from Lake Wabedo (46°54′N, 94°14′W) and Six Mile Lake (47°18′N, 94°09′W). Neither of these lakes currently supports populations of other *Coregonus* species. Table 1 outlines some important characteristics of each lake.

All study and voucher specimens were preserved in neutralized 10% formaldehyde, stored in 40% isopropanol, and were catalogued in the fish collection of the James Ford Bell Museum of Natural History, University of Minnesota.

Spawning adult fish were collected from Ten Mile lake with 1/4 inch (6.35 mm) mesh trap nets on October 19, 1984 as a source of gametes for embryo culture and chromosome analysis. Specimens for meristic, morphometric, and mtDNA analysis were collected by seine on October 12, 1987. From this later sampling 52 intact fish were sealed in plastic bags, flash frozen, and stored for up to 1 mo at −80°C prior to further processing. Gonads and hearts were dissected from partially thawed carcasses for mtDNA extraction. A unique identification number was assigned to each fish and its corresponding sample at this time.

A variety of size classes of fish, including mature adults, were collected from Lake Wabedo with gill nets on June 17 and 18, 1985. Whole carcasses from the June 17 netting were preserved as voucher specimens only. Fish from the June 18 netting served as sources of tissue for subsequent mtDNA extraction. Weight and length measurements and scale samples were taken from each fish prior to dissection. Samples of liver, spleen, muscle, heart, and gonad were dissected from each of 36 fish and placed in individually labelled Nunc Cryotubes, flash-frozen in a dry ice/ethanol mixture and stored in liquid nitrogen for up to 3 yr prior to DNA extraction.

Adult fish were collected from Six Mile Lake with gill nets on November 3 and November 19, 1987. Egg, heart, or liver samples were dissected from a total of 48 fish and sealed into individually labelled sterile tubes. Tissue samples were stored on wet ice for immediate processing. A unique identification number was assigned to each fish and its corresponding sample at this time.

Chromosome Analysis

Eggs and milt were stripped from dwarf fish and dry-fertilized in plastic teaspoons. Eggs were water-hardened and cultured in sterile glass petri dishes in distilled water at 4°C. At the blastula stage, eggs were transferred to sterile 15 mL tubes and fixed in three changes of ice-cold 50% glacial acetic acid in water. Embryos were microdissected onto glass microscope slides and their cells were mechanically dispersed. Chromosomes were spread by squashing cells under firm rolling pressure from a rubber stopper, then stained with Giemsa for observation. Chromosome spreads were examined from a total of 40 embryos.

Mitochondrial DNA Purification

Procedures used for purification of mtDNA were modified from Brown et al. (1979), Gonzalez-Villasenor et al. (1986), and Billington and Hebert (1988).

Tissues were homogenized in ice-cold TEK (50 mM Tris-HC1, pH 7.5; 10 mM EDTA, pH 8.0; 1.5% w/v KC1) at a ratio of approximately 20 mL TEK/gram tissue using a Tek-Mar Tissuemizer. DEPC (diethyl pyrocarbonate, 10% solution in ethanol) was added to the homogenate at the ratio of 1 μL per mL TEK to retard nuclease degradation.

The homogenate was underlayered with a 15 mL pad of 15% sucrose in a 50 mL conical centrifuge tube and spun on a swinging bucket rotor at 1200 × *g* for 10 min. at ambient temperature. Nuclei and cellular debris formed diffuse pellets below the sucrose interphase while the mitochondria remained suspended in the upper phase. The mitochondria-rich fraction was transferred to 35 mL Oakridge tubes, diluted 1:1 with BHB (a 5:1 ratio of 10 mM naC1 in TE : 1.5 M Sucrose in TE) (TE is 10 mM Tris-HC1, pH 7.5, and 100 mM EDTA, pH 8.0), and subjected to 23 000 × *g* at 4°C for 30 min. in a BeckmanJA-20 rotor. The supernatant was discarded; the pellet was washed with BHB and re-pelleted. Mitochondria were resuspended vigorously in 1.0 mL TE, then lysed to release DNA by adding 0.125 mL 10% SDS (sodium dodecyl sulfate) with gentle mixing. Further processing of samples was as described in Billington and Hebert (1988), except that a step-gradient was employed to shorten the time required for equilibration of DNA during ultracentrifugation. Each gradient consisted of a 1.7 mL aliquot of sample (adjusted to a concentration of 0.3 mg/mL propidium iodide and a density of 1.4 g/mL with CsC1 and TE), underlayered with 2.26 mL of a 1.7 g/mL stock solution of CsC1 in TE with 0.1 mg/mL PI. Samples were subjected to 140 000 × *g* for 22–24 h on a Beckman SW 60 ti rotor, at which time the lower fluorescent band of closed, circular mtDNA was recovered by puncturing the side of the centrifuge tube with a 23 gauge hypodermic needle. Approximately 0.2 mL containing the band of mtDNA was collected, then extracted three times with water-saturated butanol to remove excess PI. Samples were dialyzed in Spectra/Por membranes (exclusion size 12–14 kilodaltons) against two changes of 1 M NaC1 in TE with neutralized Dowex AG50-X8 resin, then against three changes of dilute TE (10 mM Tris, pH 7.5; 1 mM EDTA, pH 8.0). DNA concentration of dialyzed samples was estimated by comparing the UV-fluorescent intensity of samples with known dilutions of Lambda DNA when equal quantities of both were spotted on a 0.5% agarose gel containing 0.1 μg/mL ethidium bromide.

Mitochondrial DNA Analysis

Restriction enzyme digests of 1–12 ng of DNA were made under conditions specified by the enzyme supplier, Bethesda Research Laboratories (BRL, Gaithersburg, MD). Enzymes utilized in the current study included *Bcl* I (T/GATCA), *Hind* III (A/AGCTT), and *Xba* I (T/CTAGA). After restriction digestion, samples were end-labelled with Klenow Large Fragment of *E. coli* DNA Polymerase I (Maniatis et al. 1982, p. 114–115), providing alpha ^{32}P-labelled dATP, dGTP, dCTP, and TTP as substrates.

DNA fragments were resolved by electrophoresis through either 0.6, 1, or 1.8% agarose gels in TAE (40 mM Tris-acetate, 1 mM EDTA). Electrophoresis was performed at a current of 1.8–3.0 V·cm^{-1} for 14–18 h. After electrophoresis, gels were rinsed in distilled water, then dried under vacuum (Billington and Hebert 1988). Fragments were visualized utilizing Kodak X-Omat AR X-Ray film, with exposure at room temperature for 1–24 h without intensifying screens.

Curved-line semi-log graphs were utilized to determine sizes of fragments by comparing fragment positions on the autoradiograms relative to positions of DNA fragments of known length. Fragment size standards (*Hind* III-digested Lambda DNA fragments, 1 Kilobasepair Ladder, 123 Basepair Ladder) were all obtained from BRL.

Restriction patterns for a given restriction endonuclease were assigned a capital letter in the order in which they were discovered. Furthermore, if size variants were detected within a given restriction pattern, these variants were numbered in increasing value relative to the size of the mtDNA molecule. For example, "*Bcl* I-A2" would indicate the second-smallest size variant documented within the first restriction pattern noted upon analysis of cisco mtDNA with the enzyme *Bcl* I.

A composite summary of a characteristic array of restriction patterns noted with the enzymes *Bcl* I, *Hind* III, and *Xba* I in a given individual fish is referred to as a mtDNA clonal type,

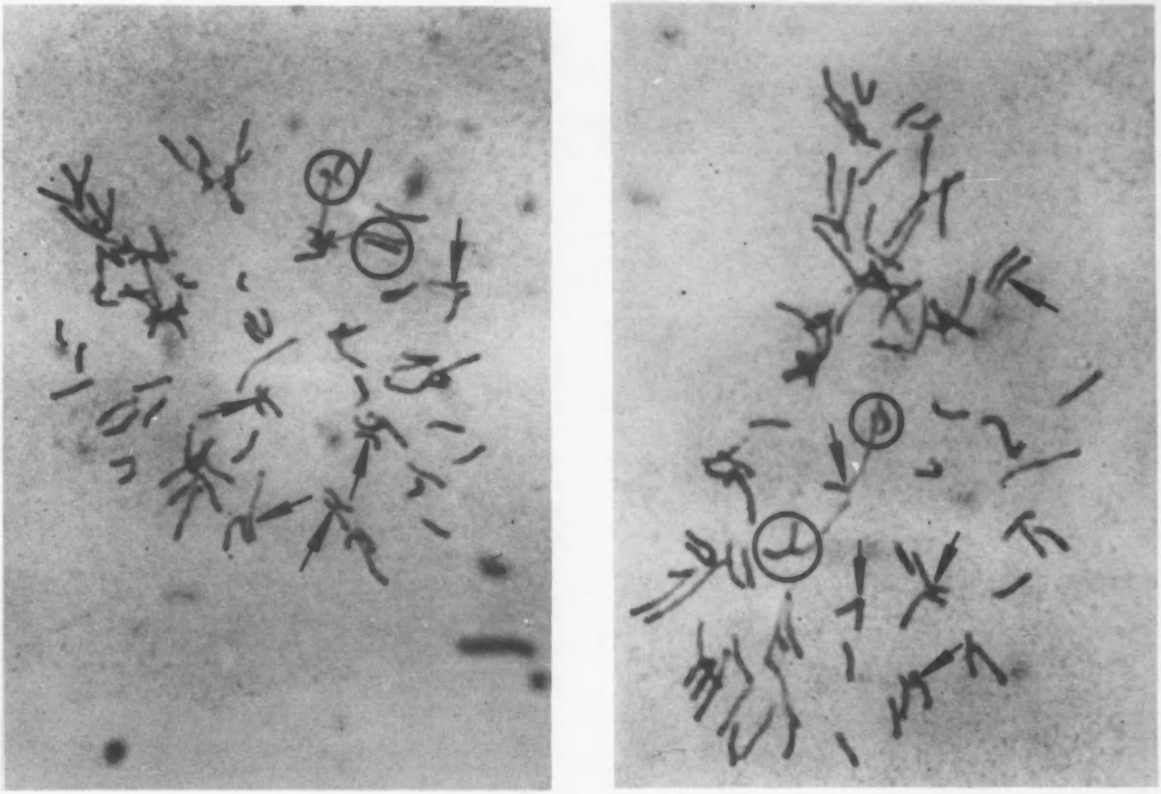

FIG. 3 and 4. Post-metaphase chromosome spreads of blastula embryos (*C. artedii*, Ten Mile Lake). Associations between chromosomes are most intimate in regions of active (dark arrows) or possible non-functional/ancestral centromeres (circled regions). The latter are inferred from associations between regions of morphologically distinct chromosomes, and may be further evidence of incomplete diploidization in *Coregonus*.

designated with capital letters in the order of their discovery. The inclusion of data on size variants resulted in the construction of a larger array of clonal types subdesignated with a number indicating the size variant.

Homology of restriction patterns between populations was confirmed by comparing restriction profiles resulting from double digests of mtDNA from individuals from each of the study populations. Intrapopulational variations was calculated as "nucleon diversity," h (equation 6, Nei and Tajima 1981) and "mean number of restriction site variations," v (equation 10, Nei and Tajima 1981). Interpopulational comparisons included "proportion of shared fragments," F (equation 21, Nei and Li 1979), "net restriction site differences between populations," d (equation 24, Nei and Tajima 1981), and the "estimated percent sequence divergence at the cleavage sites compared between populations," 100 p from (equation 3, Brown et al. 1979). Estimates of h and F were calculated both with and without data on size variants.

Results

Chromosomal Analysis

A $2n$ chromosome number of 80 has been reported for blastula embryos of *C. artedii* and most other species of *Coregonus* (Booke 1968; Gold et al. 1980; Hartley 1987). In metaphase spreads, typical centromeric associations between sister chromatids of each chromosome yielded a diploid ($2n$) chromosome value of 80 in all three study populations. However, unusual pairing of chromatids was displayed in all embryos during anaphase and early telophase of mitosis. This pairing of daughter chromatids from homologous chromosomes during the later stages of mitosis yielded an apparent diploid ($2n$) chromosome number of 40 (see Fig. 3 and 4).

Mitochondrial DNA Analysis

Figures 5–8 illustrate the restriction patterns observed with the enzymes *Bcl* I, *Hind* III, and *Xba* I. One case of heteroplasmy (involving size variation) was documented in a fish from Six Mile Lake; this individual was counted twice for population frequency estimates (Table 2).

Unexpected levels of intrapopulational diversity of mtDNA were documented in each of the study lakes; restriction pattern polymorphisms as well as discrete size variants were documented in each study population. Each of the study populations was characterized by a unique array of mtDNA clonal types

TABLE 2. Four major clonal types (A–D) were identified based on restriction site patterns alone among the three study populations; inclusion of data on size variants increased the total number of clonal types to nine.

Clonal types and size variants in study populations

Clonal type	Restriction profile			Percent frequency by lake		
	Bcl I	*Hind* III	*Xba* I	$N=48$ Six Mile	$N=52$ Ten Mile	$N=36$ Wabedo
A	A	A	A	27.1	75	19.4
A-1	A	A1	A	6.3	67.3	19.4
A-2	A	A2	A	20.8	7.7	—
B	A	A	B	72.9	3.8	—
B-1	A	A1	B1	41.7	3.8	—
B-2	A	A2	B2	22.9	—	—
B-3	A	A3	B3	8.3	—	—
C	B	A	A	—	19.3	80.6
C-1	B1	A1	A	—	13.5	72.2
C-2	B2	A2	A	—	5.8	5.6
C-3	B3	A3	A	—	—	2.8
D	B	A	B	—	1.9	—
D-1	B1	A1	B1	—	1.9	—

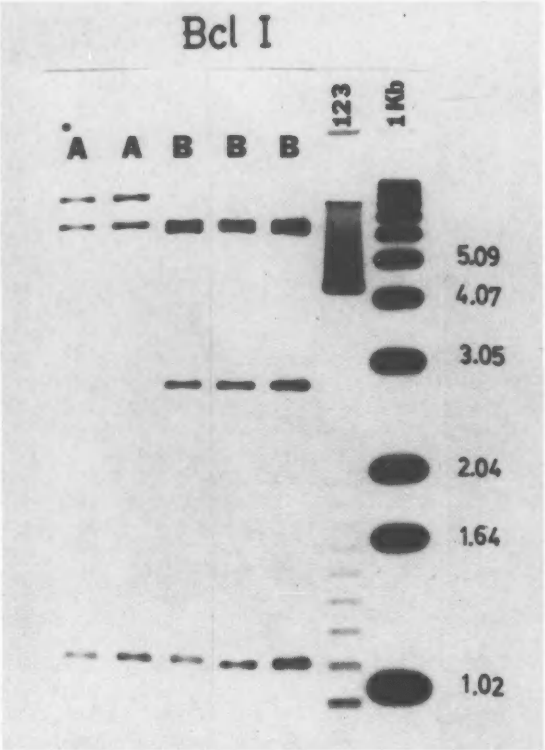

FIG. 5. Samples of mtDNA digested with *Bcl* I and subjected to electrophoresis through 1.8% agarose. Autoradiogram reveals two basic restriction patterns, a 3-banded form (A) and a 4-banded form (B). The two largest bands in the "B" pattern co-migrate under these conditions, appearing as a single, thick band of DNA.

which were present in variable frequencies (Table 2). Values of both *h* and *v* are minimal estimates, calculated from clonal types generated with only three restriction endonucleases, yet were quite high for isolated inland populations of fish (for example see Billington and Hebert 1988). Comparisons of estimates of *h* obtained from clonal types alone versus *h* obtained with the inclusion of data on size variants indicate that discrete size variants accounted for nearly half of the estimated nucleon diversity observed in cisco in Six Mile Lake (Table 3). The population in Ten Mile Lake had the highest overall levels of intrapopulational diversity of mtDNA, and encompassed all of the clonal types (and all but one of the size variants) documented in the two other study lakes.

Estimates of the percent sequence divergence (100 *p*) between the two populations of normal cisco were higher than between the dwarf fish in Ten Mile Lake and either one of the normal populations (see Table 4). Similarly, the two populations of normal-sized cisco shared less in common with each other than they each did with the population of dwarf fish. These data suggest genetic relationships between the three populations as illustrated in Fig. 9.

Discussion

As obligate oligostenotherms, *Coregonus* species are restricted to waters which provide the high oxygen and low temperature ranges suitable for their survival (Scott and Crossman 1973). Their dispersal within a water system is limited to seasons of the year when the water temperatures and flow rates of interconnecting rivers and streams are conducive to such movement; periods of drought and subsequent water temperature elevation may result in severe summer kills of cisco in marginal habitats such as Wabedo and Six Mile Lake (pers. observ.). For these reasons, populations of *Coregonus* persist in greater genetic isolation than many temperate freshwater species of fish, and headwater lakes tend to show a considerable degree of isolation from other populations within a watershed (Svaerdson 1979). Dispersal tends to be downstream, possibly as fry drifting in lake outflow during springtime higher water, as lacustrine species of *Coregonus* like ciscoes are poor at colonizing in an upstream direction (Svaerdson 1979).

Recent reductions in effective population size (or genetic "bottlenecks") from severe summer kills would, in the absence of immigration, be expected to result in reduced intrapopulational diversity of mtDNA (Avise et al. 1984; Gyllensten and Wilson 1987). Intrapopulational diversity of mtDNA clonal types (excluding size variants) was positively correlated to lake size, analogous to the findings of Vuorinen et al. (1981) in estimates of nuclear heterozygosity, and in concordance with predictions of mtDNA diversity by Avise et al. (1984). However, the levels of intrapopulational diversity observed in each population of cisco were surprisingly high in light of the biological requirements of the species. Furthermore, the mtDNA diversity in Ten Mile Lake fish probably represents the persistence of clonal types that were originally present in the colonizing (ancestral) population rather than recolonization events from downstream populations into this headwater lake.

Intrapopulational variability of mtDNA of the degree revealed in the present study, including heteroplasmy and size variation, is uncommon in vertebrates (Densmore et al. 1985; Moritz and Brown 1987; Moritz et al. 1987). The most frequently reported mtDNA polymorphisms are restriction frag-

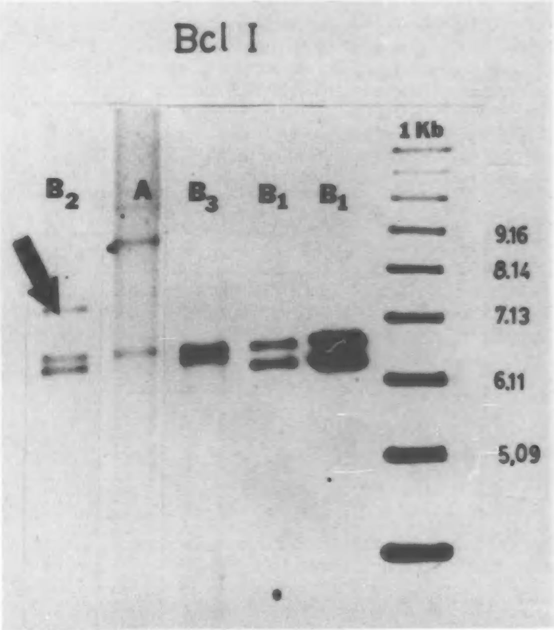

FIG. 6. Samples of mtDNA digested wtih *Bcl* I after electrophoresis through 0.6% agarose. Autoradiogram reveals the resolution of three distinct variations in the size of the second-largest fragment in pattern "B." Arrow indicates a band resulting from incomplete digestion.

ment pattern variations that result from the gain or loss of restriction enzyme recognition sites (Billington and Hebert 1988). Since the same discrete size variants were documented in three different mtDNA clonal types and were found in all three study populations, mtDNA size variations in cisco may have arisen as several independent events. The functional or evolutionary significance of such size variations is unknown, although several hypotheses on the origin of discrete length variants have been proposed (Moritz et al. 1987).

Some of the most striking cases of intrapopulational diversity of mtDNA have been reported for fish species with a long evolutionary history, such as *Amia calva* (Bermingham et al. 1986). It is unlikely that the degree of variation documented in populations of *C. artedii* in the present study indicates an unusually rapid rate of mutation in the mtDNA of this species. A more likely explanation would involve colonization of this watershed by a variety of female lineages from a pre-existing, widely distributed, long-diversified population. The latter scenario gains support from the conclusions reached by Svaerdson (1979) based on morphological evidence from Scandinavian populations of *Coregonus*. The founder effect alone could account largely for the unique array of mtDNA types observed in each population. Stochastic events and genetic drift, important factors in smaller populations, may have resulted in the loss of some of the mtDNA clonal types which were originally present in a given population at the time of colonization, and may explain the predominance of a single clonal type of mtDNA in each of the study populations (Avise et al. 1984).

Heteroplasmy, in which two or more mtDNA types occur in a given cell (or tissue) of an individual, was documented in separate extractions from the heart and ovaries of a single individual in the Six Mile Lake population. Although heteroplasmy has been documented in some homoeotherm vertebrates (Boursot et al. 1987), the majority of cases have been reported in ectotherms (Monnerot et al. 1984; Densmore et al. 1985; Bermingham et al. 1986; Wallace 1987). Most instances of heteroplasmy involve the occurrence of two discrete size variants within an individual, and this was the only type of heteroplasmy documented in the present study. Clark (1988) provided a comprehensive review of the phenomenon of mtDNA heteroplasmy.

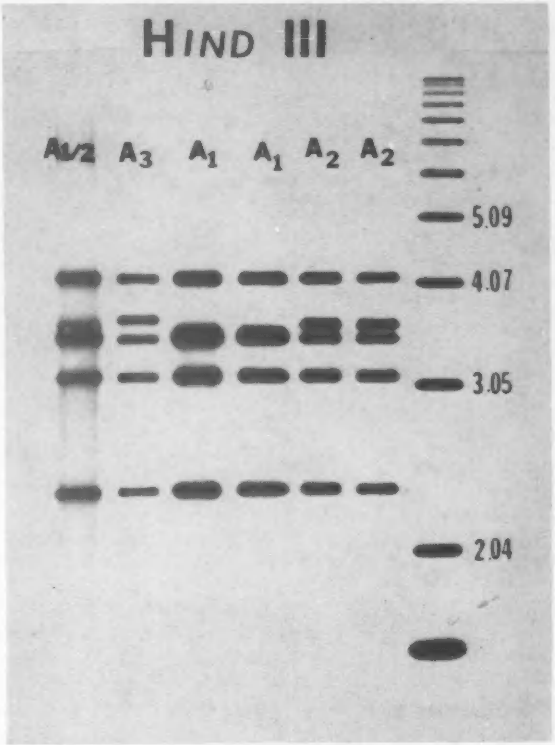

FIG. 7. Samples of mtDNA digested with *Hind* III after electrophoresis in 1% agarose. Autoradiogram reveals six fragments in all samples (smallest fragment, approximately 200 bp not shown here). Note the size variation in one of the larger bands, plus one heteroplasmic individual.

Interpopulational variation of mtDNA is more pronounced in species with a wide geographic distribution, such as the American shad (Bentzen et al. 1988), or a disjunct distribution and resultant isolated or relict populations, such as cutthroat trout (Wilson et al. 1985; Allendorf et al. 1987). The interpopulational diversity of mtDNA clonal type profiles observed in the three study populations may parallel the unique combination of meristic counts and morphometric measures characteristic of a given population of *C. artedii* (Hile 1936, 1937; B. A. Shields and J. C. Underhill, unpubl. data). Estimated divergence of mtDNA types between cisco populations in the current study approach values estimated from the mtDNA of subspecies of trout (Allendorf et al. 1987), and provide further evidence which suggest that each of the study populations within the watershed currently exist in relative genetic isolation from one another.

The unusual chromosome pairing noted in blastula embryos may be the "chromosome disturbances" noted by Svaerdson

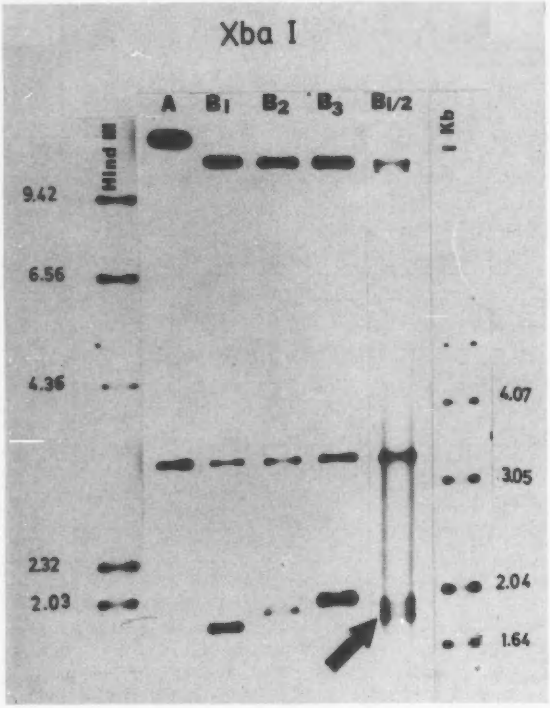

FIG. 8. Samples of mtDNA digested with *Xba* I after electrophoresis in 0.6% agarose. Autoradiogram reveals two restriction site variants, "A" and "B." Three distinct size variants occur within the smallest fragment of the "B" pattern. Note the case of heteroplasmy (arrow).

TABLE 3. Intrapopulational diversity was estimated for each of the study populations from data in Table 2 as nucleon diversity, *d*, and as the mean number of restriction site variations, *v*. Estimates of *d* were calculated both without and with considering size variants as unique morphs. The estimates of nucleon diversity were increased when data including size variations were utilized in the calculations. Note that the highest overall diversity of mtDNA types occurred in the population of dwarf fish.

Intrapopulational diversity		
Population	*d*/ + size	*v*
Ten Mile	0.406/0.528	0.224
Six Mile	0.404/0.735	0.202
Wabedo	0.311/0.450	0.161

(1979) in his studies of embryos of European *Coregonus*. Chromosome pairing was observed between morphologically distinct chromosomes in the region of what may be non-functional centromeres, remnants from past Robertsonian fusion events (Ohno et al. 1965). Such associations apparently do not occur in differentiated tissues of *Coregonus* (Sola et al. 1989). Although distributive pairing is common during meiosis in most organisms, its function in mitotic division in *Coregonus* embryos is unknown. No evidence was found in the present study to suggest that the dwarf cisco of Ten Mile Lake possesses a compliment of chromosomes unique from *C. artedii* or any other North American species of *Coregonus* (*Leucichthys*).

TABLE 4. Interpopulational comparisons were based on estimates of *F* (proportion of shared fragments), *d* (net restriction site differences), and 100*p* (estimated percent sequence divergence at cleavage sites). *F* was calculated both with and without consideration of size variants; *d* and 100*p* were both calculated on data exclusive of size variants. In all estimates, the dwarf cisco shows less divergence from (and greater similarity to) the two populations of normal-sized cisco than exists between the two normal populations.

Interpopulational comparisons			
Populations compared	*F*/(+size)	*d*	100*p*
Six Mile:Wabedo	0.8462/0.8125	1.353	2.78
Ten Mile:Six Mile	0.9286/0.8750	0.704	1.23
Ten Mile:Wabedo	0.9286/0.8824	0.541	1.23

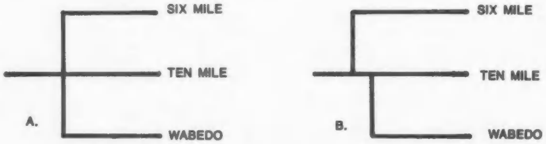

FIG. 9. Possible relationships of the three study populations based on interpopulational comparisons of major mtDNA clonal types exclusive of size variation. Diagram "A" was constructed from values of *F* and 100*p*, which indicate the population of dwarf cisco shares a like degree of genetic identity with each of the other cisco populations. Diagram "B" was constructed from estimates of *d*, which indicates a closer relationship between Ten Mile and Wabedo fish. Neither scenario suggests the dwarf fish comprise an outlier group distinct from the two normal populations of *C. artedii*.

Conclusions

Neither chromosomal nor mtDNA data from the present study indicate an evolutionary history of the dwarf fish that has been long-separated from other populations of normal *C. artedii* in the immediate watershed. It is likely that the population of dwarf cisco in Ten Mile Lake represents an extreme case of morphometric and life history variation in this phenotypically plastic and variable species complex. The intrapopulational variation in mtDNA documented in this study was unanticipated. Since mtDNA differences were documented between populations, breeding populations in each lake should be regarded as separate stocks if the management objective is to retain the natural genetic integrity of managed stocks of this species.

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References

ALLENDORF, F., N. RYMAN, AND F. UTTER. 1987. Genetics and fishery management: past, present, and future, p. 1–20. In N. Ryman, and F. Utter [ed.] Population Genetics and Fishery Management. University of Washington Press, Seattle, WA.

Avise, J. C., J. E. Neigel, and J. Arnold. 1984. Demographic influences on mitochondrial DNA lineage survivorship in animal populations. J. Mol. Evol. 20: 99–105.

Bentzen, P., W. C. Leggett, and G. G. Brown. 1988. Length and restriction site heteroplasmy in the mitochondrial DNA of American Shad (*Alosa sapidissima*). Genetics 118: 509–518.

Bermingham, E., T. Lamb, and J. C. Avise. 1986. Size polymorphism and heteroplasmy in the mitochondrial DNA of lower vertebrates. J. Hered. 77: 249–252.

Billington, N., and P. Hebert. 1988. Mitochondrial DNA variation in Great Lakes walleye (*Stizostedion vitreum*) populations. Can. J. Fish. Aquat. Sci. 45: 643–654.

Birkey, C.W., Jr., T. Maruyama, and P. Fuerst. 1983. An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. Genetics 103: 513–527.

Booke, H. E. 1968. Cytotaxonomic studies of the coregonine fishes of the Great Lakes, U.S.A.: DNA and karyotype analysis. J. Fish. Res. Board Can. 25: 1667–1687.

Boursot, P., H. Yonekawa, and F. Bonhomme. 1987. Heteroplasmy in mice with deletion of a large coding region of mitochondrial DNA. Mol. Biol. Evol. 4: 46–55.

Brown, W. M., M. George Jr., and A. C. Wilson. 1979. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. U.S.A. 76: 1967–1971.

Clark, A. G. 1988. Deterministic theory of heteroplasmy. Evolution 42: 621–626.

Densmore, L. D., J. W. Wright, and W. M. Brown. 1985. Length variation and heteroplasmy are frequent in mitochondrial DNA from parthenogenic and bisexual lizards (genus *Cnemidophorus*). Genetics 110: 689–707.

Fenderson, O. C. 1964. Evidence of subpopulations of lake whitefish, *Coregonus clupeaformis*, involving a dwarfed form. Trans. Am. Fish. Soc. 93: 77–94.

Gold, J. R., W. J. Karel, and M. R. Strand. 1980. Chromosome Formulae of North American Fishes. Prog. Fish-Cult. 42: 10–23.

Gonzalez-Villasenor, L. I., A. M. Burkhoff, V. Corces, and D. A. Powers. 1986. Characterization of cloned mitochondrial DNA from the teleost *Fundulus heteroclitus* and its usefulness as an interspecies hybridization probe. Can. J. Fish. Aquat. Sci. 43: 1866– 1872.

Gyllensten, U., and A. C. Wilson. 1987. Mitochondrial DNA of salmonids: inter- and intraspecific variability detected with restriction enzymes. p. 301–314. In N. Ryman and F. Utter [ed.] Population genetics and fishery management. University of Washington Press, Seattle, WA.

Hallerman, E. M., and J. S. Beckman. 1988. DNA-level polymorphism as a tool in fisheries science. Can. J. Fish. Aquat. Sci. 45: 1075–1097.

Hartley, S. E. 1987. The chromosomes of Salmonid fishes. Biol. Rev. 62: 197–214.

Hile, R. 1936. Summary of investigations on the morphometry of the cisco, *Leucichthys artedi* (LeSueur), in the lakes of northeastern highlands, Wisconsin. Pop. Mich. Acad. Sci., Arts, Lett. 21: 619–634.

1937. Morphometry of the cisco, *Leucichthys artedi* (LeSueur), in the lakes of the northeastern highlands, Wisconsin. Int. Rev. Gesamt–Hydrobiol. Hydrograph. 36: 57–130.

Imhof, M., R. Leary, and H. E. Booke. 1980. Population or stock structure of lake whitefish, *Coregonus clupeaformis*, in northern Lake Michigan as assessed by isozyme electrophoresis. Can. J. Fish. Aquat. Sci. 37: 783–793.

Kapuscinski, A., and D. Philipp. 1988. Fisheries genetics: issues and priorities for research and policy development. Fisheries 13: 4–10.

Lindsey, C. C., J. W. Clayton, and W. G. Franzin. 1970. Zoogeographic problems and protein variation in the *Coregonus clupeaformis* whitefish species complex. p. 127–146. *In* C. C. Lindsey and C. S. Woods [ed.] Biology of Coregonid Fishes. University of Manitoba Press, Winnipeg, Man., Canada.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Springs Harbor Laboratory, Cold Springs Harbor, New York, NY. 545 p.

Mann, G. J., and P. J. McCart. 1981. Comparison of sympatric dwarf and normal populations of least cisco (*C. sardinella*) inhabiting Trout Lake, Yukon Territory. Can. J. Fish. Aquat. Sci. 38: 240–244.

Monnerot, M., J. C. Mounolou, and M. Solignac. 1984. Intra-individual length heterogeneity of *Rana esculenta* mitochondrial DNA. Biol. Cell 52: 213–218.

Moritz, C., and W. M. Brown. 1987. Tandem duplications in animal mitochondrial DNAs: variation in incidence and gene content among lizards. Proc. Natl. Acad. Sci. U.S.A. 84: 7183–7187.

Moritz, C., T. Dowling, and W. M. Brown. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Ann. Rev. Ecol. Syst. 18: 269–292.

Nei, M., and W.- S. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. 76: 5269–5273.

Nei, M., and F. Tajima. 1981. DNA polymorphism detectable by restriction endonucleases. Genetics 97: 145–163.

Nikolsky, G., and Yu. Reshetnikov. 1970. Systematics of coregonid fishes in the USSR: intraspecies variability and difficulties in taxonomy. p. 251–266. *In* C. C. Lindsey and C. S. Woods [ed.] Biology of coregonid fishes. University of Manitoba Press, Winnipeg, Man., Canada.

Ohno, S., C. Stenius, E. Gaisst, and M. T. Zenzes. 1965. Postzygotic chromosomal rearrangements in rainbow trout (*Salmo irrideus* Gibbons). Cytogenetics 4: 117–129.

Scott, W. B., and E. J. Crossman. 1973. Freshwater fishes of Canada. Bull. Fish. Res. Board Canada. 184: 966 p.

Smith, G. R., and T. N. Todd. 1984. Evolution of species flocks of fishes in northern temperate lakes, p. 45–68. *In* I. Kornfield and A. A. Echelle [ed.] Evolution of fish species flocks. University of Maine at Orono Press, Orono, ME.

Sola, L., G. Natili, and S. Cataudella. 1989. Chromosome variability in introduced whitefishes (Genus *Coregonus*) from two lakes in central Italy. Copeia 1: 189–192.

Steinmann, P. 1951. Monographie der schweitzerischen Koregonen. Beitrag zum Problem der Entstehug vom neuen Arten. Schweiz. Z. Hydrol. 13: 54–155.

Svaerdson, G. 1970. Significance of introgression in Coregonid evolution. p. 33–60. In C. C. Lindsey and C. S. Woods [ed.] Biology of coregonid fishes. University of Manitoba Press, Winnipeg, Man., Canada.

1979. Speciation of Scandinavian *Coregonus*. Rep. Inst. Freshwater Res. Drottningholm 57: 95 p.

Todd, T. N., G. R. Smith, and L. E. Cable. 1981. Environmental and genetic contributions to morphological differentiation in ciscoes (*Coregoninae*) of the Great Lakes. Can. J. Fish. Aquat. Sci. 38: 59–67.

Vuorinen, J., M. Himberg, and P. Lankinen. 1981. Genetic differentiation in *Coregonus albula* (L.) (Salmonidae) populations in Finland. Hereditas 94: 113–121.

Wallace, G. P. 1987. Mitochondrial DNA insertion polymorphism and germ line heteroplasmy in the *Triturus cristatus* complex. Heredity 58: 229–238.

Wilson, G. M., W. K. Thomas, and A. T. Beckenbach. 1985. Intra- and inter- specific mitochondrial DNA sequence divergence in *Salmo*: rainbow, steelhead and cutthroat trouts. Can. J. Zool. 63: 2088–2094.

Zumberge, J. 1952. The lakes of Minnesota: their origin and classification. Minn. Geol. Surv. Bull. 35: 99 p.

Comparison of Logistic Regression and Discriminant Analyses for Stock Identification of Anadromous Fish, with Application to Striped Bass (*Morone saxatilis*) and American Shad (*Alosa sapidissima*)

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We examined the applicability of logistic regression to stock identification studies and compared its performance on two data sets to that of linear and quadratic discriminant functions. Logistic regression can be used to model a categorical dependent variable associated with continuous or discrete independent variables, and is preferred to discriminant analyses when the explanatory variables are not multivariate normal. Our examples were American shad (*Alosa sapidissima*) from the Connecticut River and Hudson River estuaries, and striped bass (*Morone saxatilis*) from the Hudson River, Chesapeake Bay, and Roanoke River estuaries. In the examples, we used a resampling method to assess classification and allocation errors of the two methods on new data. For the shad data, the logistic model classified significantly more fish correctly, and provided a significantly better estimate of stock composition. For the striped bass data, the two methods classified about the same proportion of fish correctly, but the logistic model gave a significantly better estimate of stock composition.

On examine l'applicabilité d'une analyse de régression logistique aux études d'identification des stocks et on compare les résultats obtenus de deux séries de données à ceux générés par des fonctions discriminantes linéaire et quadratique. L'analyse de régression logistique peut servir à modeliser une variable catégorique dépendante associée à des variables indépendantes continues ou discrètes. On la préfère aux analyses discriminantes quand les variables prédictives n'ont pas une distribution normale multivariée. Comme exemples, les auteurs ont utilisé l'alose savoureuse, *Alosa sapidissima*, des estuaires des rivières Connecticut et Hudson et le bar rayé, *Morone saxatilis*, des estuaires des rivières Hudson et Roanoake et de la baie Chesapeake. Ils se sont servis d'un échantillonnage répété pour évaluer les erreurs de classification et de répartition des nouvelles données. Pour ce qui est des données sur l'alose savoureuse, l'analyse de régression logistique a correctement classé un plus grand nombre de poissons et a fourni une meilleure estimation de la composition du stock. Dans le cas des données sur le bar rayé, les deux méthodes ont correctement classé environ le même nombre de poissons mais l'analyse de régression logistique a donné une estimation nettement supérieure de la composition du stock.

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Stock identification, the assignment of fishery resources to unit stocks, is an important component of fish population dynamics (Pitcher and Hart 1982; Cushing 1975). Included in stock identification is stock composition analysis, the classification of samples of fish into a known number of possible stocks of origin. The fisheries literature includes numerous stock composition studies, most enploying some form of discriminant function analysis (e.g. Hill 1959; Berggren and Lieberman 1977; Fabrizio 1987). In this paper, we describe and demonstrate the use of logistic regression (both dichotomous and polytomous) as an alternative to discriminant analysis for classification of fish stocks. The method has desirable statistical properties, and maximum likelihood parameter estimates are readily available from standard statistical software packages, including SAS (SAS 1987) and SYSTAT (Steinberg 1985).

Stock composition analysis is frequently performed by estimating discriminant functions on fish of known origin and subsequently using the functions to assign probabilities of stock membership to fish of unknown origin. Among the assumptions of linear discriminant analysis are that the populations are multivariate normal with different mean vectors of variates, but equal dispersion (variance-covariance) matrices. Under these conditions, linear discriminant analysis is the optimal classifier (Press and Wilson 1978; Lachenbruch 1975). The use of the quadratic discriminant function relaxes the assumption of equal covariances, but not the assumption of multivariate normality (Kendall et al. 1983; Misra 1985). However, quadratic discriminant analysis is more sensitive to sampling variation (Kendall et al. 1983). Other discriminant functions, such as growth-invariant discriminant functions, have been used (Burnaby

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1966; Fabrizio 1987); these also assume multivariate normality. As discriminant analysis has been applied many times and is well accepted in fisheries work, we omit further details here, and refer the reader to Lachenbruch (1975) or a text in multivariate statistics, such as Johnson and Wichern (1988).

Regression analysis on a categorical dependent variable is a different problem from classification, but methods developed for such regressions can be used for classification. Indeed, when discriminant functions are used to predict probabilities of class membership, they can be considered such regressions. When considered apart from the classification problem, regression with a categorical dependent variable and continuous or discrete independent variates is commonly performed by either probit or logit analysis. The underlying probability model for probit analysis is the normal distribution, while that for logit model is the logistic distribution. In practice, the two are so similar as to yield almost identical results; however, the logit model is simpler computationally. While this is of little importance in the dichotomous case, it becomes quite important in the extension to the polytomous case, where the logit model is used almost exclusively (Mantel 1966; Nerlove and Press 1973; Aldrich and Nelson 1984). In this paper, we demonstrate that logistic regression, when estimated by the method of maximum likelihood (ML), provides a useful statistical model for stock composition analysis.

Logistic Model for Classification

The logistic model can be described relatively simply. The following material draws on the work of Nerlove and Press (1973) and Aldrich and Nelson (1984). We begin with the dichotomous model, in which a number of objects (fish) may each belong to one of two classes (stocks). The class membership of fish i is represented by Y_i, a Bernoulli variable (i.e. one which assumes only the values 0 or 1). When the fish belongs to the first stock, $Y_i \equiv 0$; when it belongs to the second, $Y_i \equiv 1$. Also associated with fish i are observations on $K - 1$ continuous or discrete variables: x_{ik}, $k \in \{2, . . ., K\}$. These variables are characteristics of the fish, or features, thought to be indicative of stock membership. Examples of suitable features are morphometric, meristic, scale-shape, and protein-electrophoretic data. A constant term indexed by $k = 1$ is usually included in the model by defining $X_{i1} = 1$ for all i.

Under the dichotomous logistic model, the probability that fish i belongs to the second stock (i.e. the stock indicated by $Y_i = 1$) is estimated by

$$(1a) \quad \hat{P}_i \equiv \hat{P}(Y_i = 1) = \frac{\exp(Z_i)}{1 + \exp(Z_i)},$$

where

$$(1b) \quad Z_i \equiv \sum_{k=1}^{K} b_k X_{ik}.$$

The b_k are the K parameters of the model, one for each feature and one for the constant term. The values of the b_k are estimated by maximum likelihood, as described, e.g. in Nerlove and Press (1973).

Motivation for the form of the logistic model can be obtained by considering a simpler model, the linear probability model:

$$(2) \quad \hat{P}_i = Z_i.$$

This simple model can be fit by ordinary least squares, but has several undesirable properties. Most importantly, the left-hand side of equation (2) must lie between 0 and 1, while the right-hand side is unbounded. This anomaly in itself makes equation (2) a poor probability model. In addition, it causes the residuals of the model to be heteroscedastic; their variances change in a systematic way with $\hat{P}_i$, making parameter estimates quite sensitive to the distribution of the values in a particular sample.

How might we modify equation (2) to reconcile the bounded left-hand side with the unbounded right-hand side? The upper bound on the left-hand side can be eliminated by modeling the ratio of P_i to $(1 - P_i)$, rather than modeling P_i itself; the ratio will range from zero to infinity. The lower bound can be eliminated by taking the logarithm of the ratio, which results in the model.

$$(3) \quad \log \frac{\hat{P}_i}{1 - \hat{P}_i} = Z_i.$$

Each side of equation (3) is unbounded. When the equation is solved for $\hat{P}_i$, the result is the logistic model of equation (1a), in which each side is bounded by 0 and 1 (Fig. 1). Use of the logistic rather than the linear model for probabilities generally results in a better and more robust fit (Nerlove and Press 1973). The logistic model is empirically useful and mathematically tractable; it has also been justified on theoretical grounds by Truett et al. (1967).

Polytomous Logistic Regression

The logistic model can be readily extended from the dichotomous (two-stock) case to the polytomous (many-stock) case (Mantel 1966; Nerlove and Press 1973). In the extension to J stocks, the stock membership of fish i is again represented by Y_i, but here $Y_i \in$ "is a member of" $\{1, 2, . . ., J\}$. (Note that 0 is not a possible value.) To develop the model, let $P(Y_i = j)$, the probability that fish i belongs to the j^{th} stock, be denoted P_{ij}. Taking stock J as a baseline, we can write a model, analogous to equation (3), of the ratio of the probabililty of membership in stock j, $j \in \{1, 2, 3, . . ., J - 1\}$, to the probability of membership in stock J:

$$(4a) \quad \log (\hat{P}_{ij} / \hat{P}_{iJ}) = Z_{ij},$$

where

$$(4b) \quad Z_{ij} \equiv \sum_{k=1}^{K} b_{jk} X_{ik},$$

These are straightforward extensions of equations (3) and (1b), but here the parameters b_{jk} carry an additional subscript denoting stock membership. Solving equation (4a) for $\hat{P}_{ij}$, we get

$$(5) \quad \hat{P}_{ij} = \exp(Z_{ij}) / \hat{P}_{iJ}$$

We find the denominator, $\hat{P}_{iJ}$, by noting that $\sum^{J} P_{ij}$ must equal 1. This leads to

$$(6) \quad \hat{P}_{iJ} = \frac{1}{1 + \sum_{j=1}^{J-1} \exp(Z_{ij})}$$

The polytomous logistic model defined by equations (4b), (5), and (6) can be expressed in a simpler form, due to Mantel

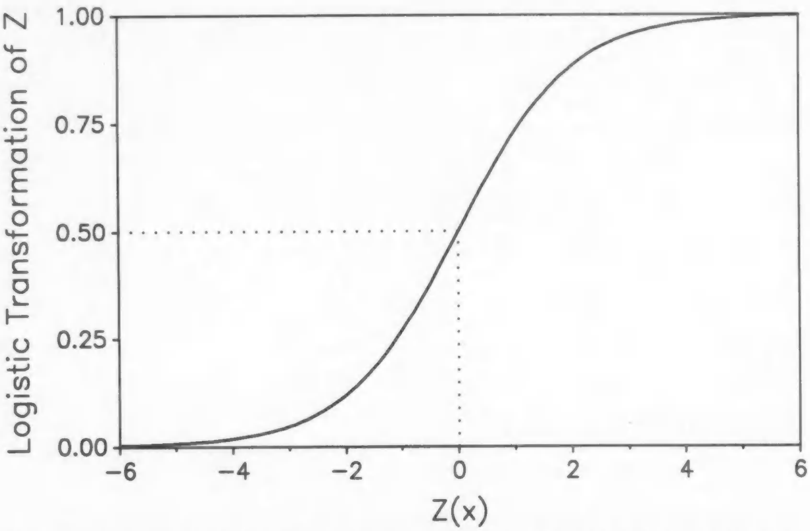

FIG. 1. Illustration of the logistic transformation, equation (1a). Although the variable Z on the horizontal axis is allowed to range from $-\infty$ to $+\infty$, the transformation (on the vertical axis) will remain between zero and one. In practice, Z is a linear combination of the feature (predictor) variables. The dotted lines illustrate the symmetry of the distribution around the point (0, 0.5).

(1966). Equation (4b) includes $K(J-1)$ parameters, where K is the number of features (including the intercept) and J is the number of stocks. (Parameter b_{jk} relates the probability of membership in stock j to the value of X_{ik}.) The simplified notation includes K additional parameters ($b_{J1}, b_{J2}, \ldots, b_{JK}$), each defined to be zero. The model can then be expressed by the single equation

$$(7) \quad \hat{P}_{ij} = \frac{\exp(Z_{ij})}{\sum_{j=1}^{J} \exp(Z_{ij})},$$

for $j \in \{1, 2, \ldots, J\}$. As in the dichotomous case, the parameters of the polytomous logistic model are readily estimated by maximum likelihood.

Comparison to Discriminant Function Analysis

In a comparison of dichotomous logistic regression to discriminant analysis, Press and Wilson (1978) enumerated arguments favoring logistic regression for classification studies. We summarize six of their arguments here: (1) When the explanatory variables for each stock are not multivariate normal, discriminant function estimators are not consistent. Thus, the prediction accuracy of a discriminant function with such data will not necessarily improve with increasing sample sizes. The logistic model, when estimated by maximum likelihood, provides consistent estimates. (2) When the normality assumption is not met, discriminant function estimation (including stepwise discriminant procedures) can give misleading information about the significance of coefficients. Meaningless variables may be included by the discriminant estimator (even with large sample sizes), but not by the ML estimator of the logistic model. (3) Halperin et al. (1971) conducted numerical comparisons of ML logistic analysis and discriminant function estimation. They found that, under non-normal conditions, the ML logistic estimator usually gives slightly better fit than the discriminant function. (4) Maximum likelihood estimation is more likely to provide warning (by failing to converge) when the model is poorly determined by the data. (5) The maximum likelihood estimators are functions of sufficient statistics, while the discriminant estimators are not. The use of sufficient statistics is associated with lower mean squared errors. (6) Discriminant analysis may produce biased estimators in some applications, in particular where the underlying process is logistic (McFadden 1976).

Applications to Data

To compare the results of logistic regression to those of discriminant analysis, we analyzed two sets of data on fish of known stocks of origin. These analyses were not intended to be definitive characterizations of the two methods, but rather to give some indication of their comparative merit on actual fisheries data. We analyzed each data set by both classification methods, and computed statistics to describe each method's accuracy in classification and allocation. The Wilcoxon signed-ranks test (a nonparametric statistical test of paired differences; Hollander and Wolfe 1973) was used to compare the two methods. The two data sets, shad and striped bass, were analyzed by identical methods, but completely separately.

Data

The first data set used for testing was collected by Hill (1959), and contained information on 310 American shad, *Alosa sapidissima*, from the Connecticut and Hudson Rivers. We used the 300 observations without missing values (Table 1). Hill's data include counts of the posterior scutes, vertebrae, pectoral rays, anal rays, and dorsal rays of each fish. Hill chose these five characters for his own analyses because he found that these features did not vary with size. We used these data to demonstrate application of the dichotomous logistic model.

TABLE 1. Summary of data used in the study. Striped bass data and collection methods are described in Fabrizio (1987); American shad data and collection methods, in Hill (1959).

Species	Number of fish	Stock of origin	Year collected
American shad	91	Connecticut River	1945
American shad	104	Hudson River	1939
American shad	105	Hudson River	1940
Total	300		
Striped Bass	86	Chesapeake Bay	1984
Striped Bass	28	Chesapeake Bay	1985
Striped Bass	34	Hudson River	1984
Striped Bass	47	Hudson River	1985
Striped Bass	98	Hudson River	1986
Striped Bass	143	Roanoke River	1984
Striped Bass	97	Roanoke River	1985
Striped Bass	92	Roanoke River	1986
Total	625		

The second data set was compiled by Fabrizio (1987) from 644 striped bass, *Morone saxatilis*, of the Hudson River, Chesapeake Bay, and the Roanoke River stocks. We omitted data on 19 fish as being apparent outliers, leaving 625 observations (Table 1). The data collected from each fish were fork length, sex, head length, jaw length, snout length, and internostril width; in addition, six variables were derived from isoelectric focussing analysis of eye lens proteins. Fabrizio (1987) subjected these six variables to the arcsine transformation prior to further analysis; we used the transformed variables.

Two difficulties arose in connection with the morphometric variables on striped bass. First, fork length was highly correlated ($r>0.9$) with each of the four other morphometric variables. Second, mean fork length differed strongly by stock of origin. This might have made it useful in classification, but because the relationship of fork length to stock of origin can be quite variable, we wished to consider only models not including body length effects. To resolve these issues, we regressed each morphometric variable against fork length, and performed a principal component analysis on the residuals. The first three principal components, which contained approximately 90% of the remaining variance, were used as features in further analyses. This procedure removed length effects, eliminated correlation among variables, and, by omitting the final principal component, allowed us to discard the portion of the variance least correlated among variables, a portion that we believed likely to contain much of the measurement error. We classified the striped bass data into the three component stocks to illustrate application of the polytomous logistic model.

Misclassification Statistics

We used two statistics to summarize the results of each classification method on each data set. The first statistic estimated the misclassification rate of each method. Lachenbruch and Mickey (1968) described and evaluated eight methods of estimating misclassification rates of discriminant functions. Three common methods that do not depend upon the assumption of normality, and thus are suitable for evaluating classification rules for non-normal data, are (1) the resubstitution method, which computes the error rate on the data used for fitting the model (2) the holdout method, which computes the error rate on a subsample of data withheld from model development; and (3) the jackknife method, in which each point is held out in turn and then classified by the rule developed on all other points.

Each of the three methods has its advantages and disadvantages. The resubstitution method yields a statistic known as the apparent error rate (Lachenbruch 1975) which, while simple to compute, tends to underestimate the error rate on new data, even when the populations are normal (Kendall et al. 1983). The holdout procedure, although not subject to the large optimistic bias of the resubstitution method, has three significant disadvantages. First, it requires a relatively large sample. Second, it evaluates a different model from the one that will be used in practice, because before the model is used in practice, it is usually recomputed on the entire data set. Finally, choosing the size of the holdout sample is problematic: a large holdout sample gives a good estimate of the model's performance, but that performance may be poor. A small holdout sample provides a better model, but a highly variable estimate of its performance.

Lachenbruch and Mickey (1968) found the jackknife to be the best of the methods not assuming normality. However, the jackknife is computationally intensive, a property which prohibited its use in the present study, given our use of an iterative maximum-likelihood procedure for estimation of the logistic models. Since the iterative solution was slow, computing jackknifed estimates of error rates would have been impractical.

To provide reasonably accurate and precise estimates of error rates, we developed a hybrid method combining elements of the holdout and jackknife methods. In the holdout method, the data are partitioned randomly into what might be called a training subset and a verification subset. In our hybrid procedure, we randomly partitioned the data not once, but 25 times. In making each partition, we allocated about 70% of the data to the training subset and the rest to the verification subset. Each of the 25 training subsets was then used to fit both a discriminant function and a logistic regression model. The parameters of each model were used to classify the observations in the corresponding verification subset.

After each of the 25 verification subsets had been classified both by logistic regression and by discriminant analysis, we evaluated the success of each classification. A measure of a model's misclassification rate on stock j is provided by the statistic E_j:

$$(8) \quad E_j = |N_j - \hat{N}_j| \,/\, N_j,$$

where N_j is the number of fish from stock j in the verification set and $\hat{N}_j$ is the number of those fish classified into the correct stock by the model in question. For comparative purposes, we used the total misclassification rate statistic E, a weighted mean of the E_j's:

$$(9) \quad E = \frac{\sum_{j=1}^{J} N_j E_j}{\sum_{j=1}^{J} N_j}.$$

The E statistic, then, provides an estimate of the expected misclassification rate of a model on new data.

Misallocation Statistics

The misclassification rate is not the only, and perhaps not the most appropriate, measure of error in stock composition analysis. Although the goal of such an analysis is to assign each fish to its correct stock, this is rarely achieved. In the absence of a perfect classification, one may be more interested in a different measure of error: the misallocation of the sample in relation to its actual stock composition. In many fishery contexts, the accuracy of the allocation (the proportion assigned to each stock) will be of more interest than the proportion of individual fish classified correctly. To quantify misallocation error, we propose the new statistic C, which is the mean of the allocation error statistics (C_j, defined below) on fish from the J individual stocks:

$$\text{(10a)} \quad C = \frac{1}{J}\sum_{j=1}^{J} C_j,$$

The allocation error of the model on fish from stock j only is represented by C_j:

$$\text{(10b)} \quad C_j = \frac{|(P_j - \hat{P}_j)|^{1.5}}{d_j}.$$

Here P_j is the true proportion of the total sample that comes from stock j, $\hat{P}_j$ is the proportion of the total sample classified into stock j by the algorithm under consideration, and d_j is a normalization factor that limits C_j to values between 0 and 1:

$$\text{(10c)} \quad d_j = \begin{cases} 1, & \text{for } \mathrm{P}_j = 1 \text{ or } P_j = 0, \\ 1 - \mathrm{P}_j, & \text{for } \hat{P}_j \geq \mathrm{P}_j, \\ \mathrm{P}_j, & \text{for } \hat{P}_j < \mathrm{P}_j. \end{cases}$$

The numerator of equation (10b) is raised to the 1.5 power to increase the penalty at a greater-than-linear rate as the error between estimated and observed proportions becomes larger. The value of this exponent was chosen heuristically, but the resulting statistic seems to provide reasonable penalties for different values of P_j and $\hat{P}_j$ (Fig. 2). Since this is so, we believe that C is a useful statistic for comparing estimated allocations.

How does the misallocation rate C compare to the misclassification rate E? When all fish are classified correctly, E and C are each zero. When some fish are misclassified, C conveys different information from E. To illustrate this point, consider a hypothetical case in which 200 fish with a true stock composition of 3:1 (150:50) are classified. A value of $E = 0.25$, reflecting an overall misclassification rate of 25% (50 fish), might be the best obtainable in a particular case. If the model misclassified exactly 25 fish from each stock (which would give $E = 0.25$), the estimate of stock composition would nonetheless be exactly correct (which would give $C = 0$). However, a value of $E = 0.25$ would also occur if all 50 fish of the smaller stock were misclassified. This would be evidence of a poor model, and the value of $C = 0.5$ would reflect that.

The values of $\hat{P}_j$ for computing C can be obtained by at least two methods. The methods differ in the computation of $\hat{N}_j$, the number of fish assigned to each stock. In the first method, $\hat{N}_j$ is the sum of the predicted probabilities that each fish belongs to stock j. In the second method, each fish is assigned to the stock for which its predicted probability of membership is highest. This method must be used in computing the misclassification statistic E, as for that purpose each fish must be classified into one stock or the other. In computing the misallocation statistic C, however, one can use the first method, which distinguishes a very certain classification (where one of the predicted probabilities approaches unity) from an uncertain one (where the predicted probabilities are nearly equal). In this paper we used the first method to compute C.

We used standard software for most of the analyses. The parameters of the logistic model for each of the 25 partitions of each species were estimated with the CATMOD procedure of SAS (SAS 1987). To classify the corresponding verification set for each partition, we used a short computer program (written in FORTRAN) to implement equation (7) and provide summary statistics. The discriminant functions were computed by

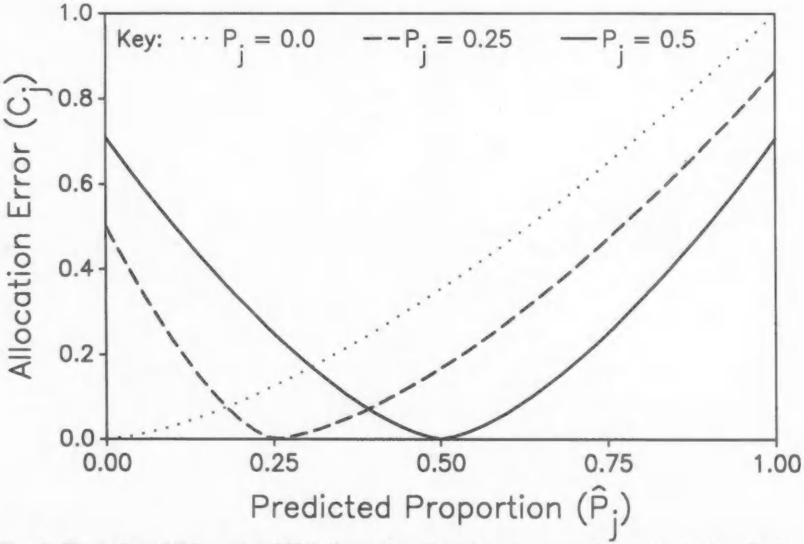

FIG. 2. Illustration of C_j [equation (10b)], the portion of the allocation error C occurring in classification of fish from stock j. The three curves represent values of C_j over all $\hat{P}_j$ for the three true values $P_j = 0.0$, $P_j = 0.25$, and $P_j = 0.5$. The curve for any $P_j = y$ $(0 \leq y \leq 1)$ is a mirror image of the curve for $P_j = 1 - y$.

TABLE 2. Comparison of discriminant analysis and logistic regression results in classifying American shad and striped bass. An asterisk (*) indicates statistically significant differences in misallocation (C statistic) or misclassification (E statistic) at $\alpha = 0.05$ by Wilcoxon's signed-rank test.

Error statistic	Mean for logistic regression ($N=25$)	Mean for discriminant analysis ($N=25$)	Number of cases in which LR classifier was superior	Probability that methods are equally good classifiers
American shad				
C	0.0213	0.395	19	0.015*
E	0.1496	0.1704	19[a]	0.003*
Striped bass				
C	0.0106	0.0218	20	< 0.002*
E	0.2500	0.2401	8[b]	0.121

[a] In addition, two pairs were ties.

[b] In addition, three pairs were ties.

the DISCRIM procedure of SAS (SAS 1987). The American shad data gave no indication of unequal dispersion matrices between populations, and therefore a linear discriminant function was used. For the striped bass, the data in each partition were tested for homogeneity of the dispersion matrices. In each case, the null hypothesis (of homogeneity) was rejected, and therefore a quadratic discriminant function was used.

We compared the results of the two classification methods by forming paired differences of the E and C statistics for each partition. Within each pair, both values were of either E or C; one described the logistic analysis; the other, the discriminant analysis of the same data. The sample size of 25 for each test reflected the 25 partitions of each data set. We tested at $\alpha = 0.05$ the null hypothesis that the paired differences were not different from zero. To avoid the assumptions of parametric testing, we used the Wilcoxon's signed-rank test (Hollander and Wolfe 1973).

Results

The results of the empirical study indicated that, for these data, logistic regression was a better classifier than discriminant analysis (Table 2). Logistic regression provided both lower allocation errors (C statistic; $P = 0.015$) and lower classification errors (E statistic; $P = 0.003$) on the American shad data. On the striped bass data, logistic regression provided significantly lower allocation errors ($P = 0.002$), while the two methods were not significantly different in classification error ($P = 0.12$).

The superiority of the logistic regression classifiers is also visible in the distribution of errors of each type. Figures 3 and 4 illustrate the distribution of allocation errors and classification errors on the American shad data, while Fig. 5 and 6 illustrate the same information on the striped bass data. Most of the largest errors were from discriminant analyses, and the logistic analyses had consistently lower errors.

Discussion

In recent years, several new laboratory techniques have been described for fish stock identification, but discriminant analysis has remained the standard statistical tool for stock composition analysis. However, discriminant analysis assumes normality and is not robust to violations of that assumption (Eisenbeis

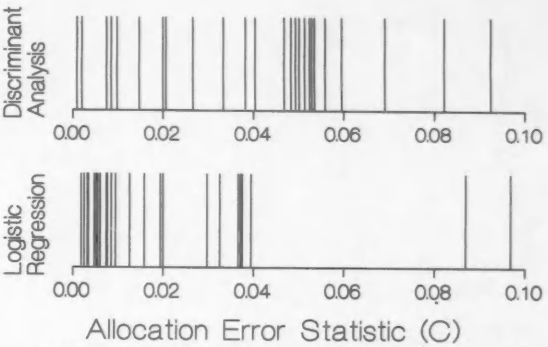

FIG. 3. Values of the allocation error statistic C observed when classifying American shad by two statistical methods. Upper stripe plot: values of C from 25 discriminant function analyses; lower plot: values of C from 25 logistic regression analyses. Each vertical line in the stripe plots represents one (or more, if equal) observed value of C. The vertical scale carries no information. Stripe plots provide the same information as histograms, but are used here because they illustrate the range of the data more distinctly.

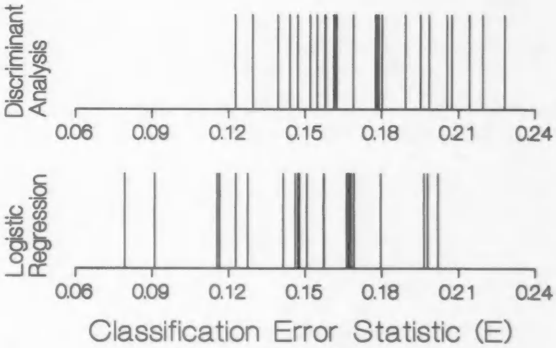

FIG. 4. Distribution of the classification error statistic E when classifying American shad by two statistical methods. Upper stripe plot: values of E from 25 discriminant function analyses; lower plot: values of E from 25 logistic regression analyses. (The caption to Fig. 3 contains more information on stripe plots.)

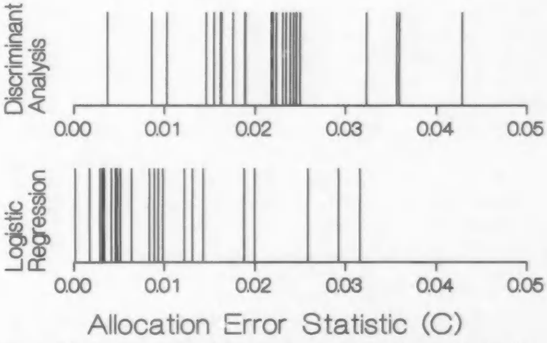

FIG. 5. Distribution of the allocation error statistic C when classifying striped bass by two statistical methods. Upper stripe plot: values of C from 25 logistic regression analyses. (The caption to Fig. 3 contains more information on stripe plots.)

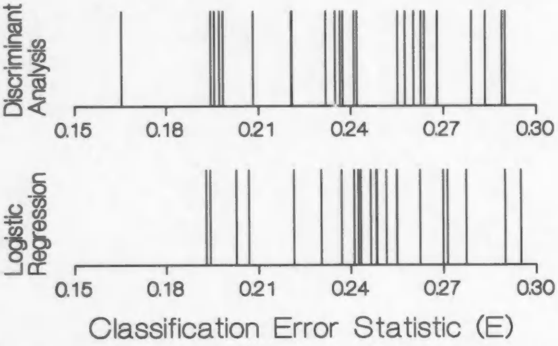

FIG. 6. Distribution of the classification error statistic E when classifying striped bass by two statistical methods. Upper stripe plot: values of E from 25 discriminant function analyses; lower plot: values of E from 25 logistic regression analyses. (The caption to Fig. 3 contains more information on stripe plots.)

1977; Krzanowski 1977). It would seem that evaluation of other methods is necessary, or at least desirable.

The logistic model described here is strictly appropriate (maximum-likelihood) for the following kinds of data (Kendall et al. 1983): (1) normal variables with equal covariances;(2) independent binary variables; (3) binary variables following the linear logistic model with second- and higher-order effects equal; and (4) a combination of (1) and (3). However, the empirical results of our study and the observations of Press and Wilson (1978) and others (e.g. Krzanowski 1975, 1977; Halperin et al. 1971) suggest that logistic regression is relatively robust to the distribution of the underlying data. Thus we would expect it to be at least as useful as, if not more useful than, discriminant analysis in many cases.

Efron (1975) demonstrated that with true multivariate normal data and large separation between populations, the asymptotic efficiency of the logistic model is less than that of discriminant analysis. Some biological variates are approximately normal; others approximate normality after logarithmic transformation. However, many data used in stock composition studies do not appear to follow any distribution strictly, and certainly many such data, such as counts and proportions, are not multivariate normal. Moreover, there is no conclusive test for multivariate normality (Johnson and Wichern 1988). In this context, our conclusion from Efron's (1975) finding is that discriminant analysis should be included among the methods of analysis when the data appear to be multivariate normal.

Beyond the method discussed here, we are aware of two recent papers that have described novel statistical methods for stock composition analysis. The first method was developed by Fournier et al. (1984), who called it FMP-LS (finite mixture problem with learning sample). That procedure uses conditional maximum likelihoods and requires assuming a specific probability density function for each feature variable. The second method, a neutral network variant called GMDH (group method of data handling), was presented by Prager (1988) as an adaptation of methods developed by Ivakhnenko et al. (1979). While either or both of these methods may prove valuable, they appear to share two drawbacks at present. First, each requires relatively complex computations for which computer programs are not readily available. Second, the statistical properties of these two methods have not been well studied. In particular, the performance of the empirical GMDH procedure of Prager (1988) will require further Monte Carlo studies to establish.

In comparison to these two methods, logistic regression appears relatively attractive. The computations for logistic regression can be performed by several widely used statistical software packages. Only the assignment of new individuals to stocks may require programming, and that computation is simple enough to be done on a hand calculator if necessary. Furthermore, since logistic regression has been the subject of many studies (e.g. Anderson 1972; Gordon 1974, Efron 1975), the statistical properties of the method are relatively well known.

While the empirical results reported here do not prove that logistic regression will be superior to discriminant analysis in any given case, they indicate that it has that potential. At this stage of our knowledge, it would be premature to suggest that fishery biologists abandon the well-known tool of discriminant analysis. We do suggest, however, that those performing stock composition analyses should evaluate more than one statistical method, and that the logistic regression model described here is worthy of inclusion.

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References

ALDRICH, J. H., AND F. D. NELSON. 1984. Linear probability, logit, and probit models. Sage Univ. Paper series on Quantitative Applications in the Social Sciences, 07–045. Sage Publications, Beverly Hills, CA.

ANDERSON, J. A. 1972. Separate sample logistic discrimination. Biometrika 59: 19–35.

BERGGREN, T. J., AND J. T. LIEBERMAN. 1977. Relative contribution of Hudson, Chesapeake, and Roanoke striped bass, *Morone saxatilis*, stocks to the Atlantic coast fishery. U.S. Fish. Bull. 76: 335–345.

BURNABY, T. P. 1966. Growth-invariant discriminant functions and generalized distances. Biometrics 22: 96–110.

CUSHING, D. C. 1975. Marine ecology and fisheries. Cambridge University Press, Cambridge, UK. 278 p.

EFRON, B. 1975. The efficiency of logistic regression compared to normal discriminant analysis. J. Am. Stat. Assoc. 70: 892–898.

EISENBEIS, R. A. 1977. Pitfalls in the application of discriminant analysis in business, finance, and economics. J. Finance 32: 875–900.

FABRIZIO, M. C. 1987. Growth-invariant discrimination and classification of striped bass stocks by morphometric and electrophoretic methods. Trans. Am. Fish. Soc. 116: 728–736.

FOURNIER, D. A., T. D. BEACHAM, B. E. RIDDELL, AND C. A. BUSACK. 1984. Estimating stock composition in mixed stock fisheries using morphometric, meristic, and electrophoretic characteristics. Can. J. Fish. Aquat. Sci. 41: 400–408.

GORDON, T. 1974. Hazards in the use of the logistic function with special reference to data from prospective cardiovascular studies. J. Chron. Dis. 27: 97–102.

HALPERIN, M., W. C. BLACKWELDER, AND J. I. VERTER. 1971. Estimation of the multivariate logistic risk function: a comparison of the discriminant function and maximum likelihood approaches. J. Chronic Dis. 24: 125–158.

HILL, D. R. 1959. Some uses of statistical analysis in classifying races of American shad (*Alosa sapidissima*). U.S. Fish. Bull. 59: 269–286.

HOLLANDER, M., AND D. A. WOLFE. 1973. Nonparametric statistical methods. John Wiley and Sons, New York, NY. 503 p.

IVAKHNENKO, A. G., G. I. KROTOV, AND V. N. VISOTSKY. 1979. Identification of the mathematical model of a complex system by the self-organization method, p. 324–352. *In* E. F. Halfon [ed.] Theoretical systems ecology. Academic Press, New York, NY.

JOHNSON, R. A., AND D. W. WICHERN. 1988. Applied multivariate statistical analysis, 2nd ed. Prentice Hall, Englewood Cliffs, NJ. 607 p.

KENDALL, M., A. STUART, AND J. K. ORD. 1983. The advanced theory of statistics, vol. 3, 4th ed. Charles Griffin, London, UK. 780 p.

KRZANOWSKI, W. J. 1975. Discrimination and classification using both binary and continuous variables. J. Am. Stat. Assoc. 70: 782–790.

1977. The performance of Fisher's linear discriminant function under non-optimal conditions. Technometrics 19: 191–200.

LACHENBRUCH, P. A. 1975. Discriminant analysis. Hafner Press, New York, NY. 128 p.

LACHENBRUCH, P. A., AND M. R. MICKEY. 1968. Estimation of error rates in discriminant analysis. Technometrics 10: 1–11.

MANTEL, N. 1966. Models for complex contingency tables and polychotomous dosage response curves. Biometrics 22: 83–95.

MCFADDEN, D. 1976. A comment on discriminant analysis "versus" logit analysis. Ann. Econ. Social Meas. 5: 511–523.

MISRA, R. K. 1985. Quadratic discriminant analysis with covariance for stock delineation and population differentiation: a study of beaked redfishes (*Sebastes mentella* and *S. fasciatus*). Can. J. Fish. Aquat. Sci. 42: 1672–1676.

NERLOVE, M., AND S. J. PRESS. 1973. Univariate and multivariate log-linear and logistic models. Report R-1306-EDA/NIH. Rand Corporation, Santa Monica, CA. 134 p.

PITCHER, T. J., AND P. J. B. HART. 1982. Fisheries ecology. Croom Helm, London, UK. 414 p.

PRAGER, M. H. 1988. Group method of data handling: a new method for stock identification. Trans. Am. Fish. Soc. 117: 290–296.

PRESS, S. J., AND S. WILSON. 1978. Choosing between logistic regression and discriminant analysis. J. Am. Stat. Assoc. 73: 699–705.

SAS INSTITUTE INC. 1987. SAS/STAT guide for personal computers, vers. 6 ed. SAS Institute, Cary, NC. 1028 p.

STEINBERG, D. 1985. Logit: a supplementary module for SYSTAT. Supplied with software purchase from SYSTAT, Inc., Evanston, IL. 60201.

TRUETT, J., J. CORNFIELD, AND W. KANNEL. 1967. A multivariate analysis of the risk of coronary heart disease in Framingham. J. Chron. Dis. 20: 511–524.

Lake Acidification and Fisheries Project: Brook Trout (*Salvelinus fontinalis*) Early Life Stages[1]

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The following seven papers represent the second of several sets planned for publication in the Canadian Journal of Fisheries and Aquatic Sciences (CJFAS) from the Electric Power Research Institute's (EPRI) Lake Acidification and Fisheries (LAF) Project. The first set of nine papers dealt with responses of adult brook trout (*Salvelinus fontinalis*) (Booth et al. 1988; McDonald and Milligan 1988; Mount et al. 1988a, b; Tietge et al. 1988; Walker et al. 1988; Wood et al. 1988a, b, c). The seven papers in this issue present toxicology, physiology, and histopathology results on brook trout eggs, fry, and juveniles exposed in the laboratory to water quality conditions typical of acidified lakes and streams, with lowered pH, elevated aluminum concentrations, and low calcium concentrations.

The overall goal of the LAF Project is to determine cause–effect relationships between surface water acidification and fish population responses by integrating bioassay data from the laboratory and water quality and fish population data from the field into a predictive modeling framework. An overview of specific steps in the project and a summary of major findings on adult brook trout are given in Bergman et al. (1988) and will not be reiterated here.

Brook Trout Early Life Stages

In the set of seven papers in this issue, LAF investigators report results from a series of experiments with brook trout eggs, fry, and juveniles exposed for as long as 91 d to as many as 84 different combinations of pH (4.0–6.5), aluminum (0–1000 μg/L), and calcium (0.5–8.0 mg/L) representative of acidic and acid-sensitive surface waters. Ingersoll et al. (1990a) show that mortality and growth effects of acid/aluminum exposure are dependent on life stage, with sensitivity to acid alone decreasing with age from freshly fertilized eggs through swim-up fry, while sensitivity to aluminum alone increases with age through the same life stages. Increasing calcium is beneficial to all life stages, with the degree of benefit dependent on life stage and acid/aluminum concentrations.

Surviving brook trout fry from Ingersoll's experiments were used by Wood et al. (1990a, b) to investigate sublethal, ionoregulatory effects of acid/aluminum/low calcium exposure for 91 d from fertilization through swim-up and for 21-d periods encompassing yolk sac and swim-up fry stages. Whole body ion concentrations, determined by neutron activation analysis, were affected most by water calcium concentration during the full 91-d early life-stage exposure, while pH was the dominant influence on whole body electrolytes during the 21-d yolk sac and swim-up fry exposures. In a related experiment lasting 40 d from the eyed egg through swim-up stages, Ingersoll et al. (1990b) noted that increased acidity caused hypertrophy and hyperplasia of epidermal mucous cells, while low calcium concentrations induced mucous cell hyperplasia. Ingersoll et al. (1990b) suggest that these acid and low calcium-induced effects on epidermal mucous cells may represent a compensatory response to counter ionoregulatory stress observed by Wood et al. (1990a, b).

The three final papers in this set add to our understanding about the effects of pulsed acid/aluminum exposure (Mount et al. 1990), effects of aluminum complexation on toxicity (Parkhurst et al. 1990), and the relative sensitivity of different brook trout strains to combinations of acid, aluminum, and low calcium concentrations (Ingersoll et al. 1990c).

The Future

Additional publications from the LAF Project will include toxicology, physiology, and histopathology results of further experiments with brook trout, rainbow trout, white sucker, and smallmouth bass, demonstrations and applications of the models and modeling framework built upon these results, and comparisons and syntheses covering results from all four fish species studied in the LAF Project. A set of papers describing the acclimation to sub-lethal aluminum exposure in brook trout and rainbow trout is currently in review at CJFAS. The LAF modeling framework is now being used for several tasks in the National Acid Precipitation Assessment Program in the United States as described by Baker et al. (1990).

Data Availability

Because of the extensive nature of the toxicology data sets being produced in this project (e.g. daily observations on as many as 84 different exposure combinations of pH, aluminum, and calcium in 17 different experiments that together cover six life stages of brook trout), only summaries and applications of data are presented in the publications in past, current, and anticipated issues of CJFAS. Because many investigators and agencies may find additional uses for the full toxicology data sets, they will be made available on computer disc or tape from the Electric Power Software Center, 1930 Hiline Drive, Dallas, TX 75207, USA.

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References

BAKER, J. P., D. P. BERNARD, S. W. CHRISTENSEN, AND M. J. SALE. 1990. Biological effects of changes in surface water acid-base chemistry. State-of-Science/Technology Report 13. National Acid Precipitation Assessment Program, Washington, DC.

BERGMAN, H. L., J. S. MATTICE, AND D. J. A. BROWN. 1988. Lake acidification and fisheries project: adult brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1561–1562.

BOOTH, C. E., D. G. MCDONALD, B. P. SIMONS, AND C. M. WOOD. 1988. Effects of aluminum and low pH on net ion fluxes and ion balance in the brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1563–1574.

INGERSOLL, C. G., D. R. MOUNT, D. D. GULLEY, T. W. LAPOINT, AND H. L. BERGMAN. 1990a. Effects of pH, aluminum, and calcium on survival and growth of eggs and fry of brook trout (*Salvelinus fontinalis*). 47: 1580–1592.

INGERSOLL, C. G., D. A. SANCHEZ, J. S. MEYER, D. D. GULLEY, AND J. E. TIETGE. 1990b. Epidermal response to pH, aluminum, and calcium exposure in brook trout (*Salvelinus fontinalis*) fry. Can. J. Fish. Aquat. Sci. 47: 1616–1622.

INGERSOLL, C. G., D. D. GULLEY, D. R. MOUNT, M. E. MUELLER, J. D. FERNANDEZ, J. R. HOCKETT, AND H. L. BERGMAN. 1990c. Aluminum and acid toxicity to two strains of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 1641–1648.

MCDONALD, D. G., AND C. L. MILLIGAN. 1988. Sodium transport in the brook trout, *Salvelinus fontinalis*: effects of prolonged low pH exposure in the presence and absence of aluminum. Can. J. Fish. Aquat. Sci. 45: 1606–1613.

MOUNT, D. R., C. G. INGERSOLL, D. D. GULLEY, J. D. FERNANDEZ, T. W. LAPOINT, AND H. L. BERGMAN. 1988a. Effect of long-term exposure to acid, aluminum, and low calcium on adult brook trout (*Salvelinus fontinalis*). 1. Survival, growth, fecundity, and progeny survival. Can. J. Fish. Aquat. Sci. 45: 1623–1632.

MOUNT, D. R., J. R. HOCKETT, AND W. A. GERN. 1988b. Effect of long-term exposure to acid, aluminum, and low calcium on adult brook trout (*Salvelinus fontinalis*). 2. Vitellogenesis and osmoregulation. Can. J. Fish. Aquat. Sci. 45: 1633–1642.

MOUNT, D. R., M. J. SWANSON, J. E. BRECK, A. M. FARAG, AND H. L. BERGMAN. 1990. Responses of brook trout (*Salvelinus fontinalis*) fry to fluctuating acid, aluminum, and low calcium exposure. Can. J. Fish. Aquat. Sci. 47: 1623–1630.

PARKHURST, B. R., H. L. BERGMAN, J. D. FERNANDEZ, D. D. GULLEY, J. R. HOCKETT, AND D. A. SANCHEZ. 1990. Inorganic monomeric aluminum and pH as predictors of acidic water toxicity to brook trout. Can. J. Fish. Aquat. Sci. 47: 1631–1640.

TIETGE, J. E., R. D. JOHNSON, AND H. L. BERGMAN. 1988. Morphometric changes in gill secondary lamellae of brook trout (*Salvelinus fontinalis*) after long-term exposure to acid and aluminum. Can. J. Fish. Aquat. Sci. 45: 1643–1648.

WALKER, R. L., C. M. WOOD, AND H. L. BERGMAN. 1988. Effects of low pH and aluminum on ventilation in the brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1614–1622.

WOOD, C. M., R. C. PLAYLE, B. P. SIMONS, G. G. GOSS, AND D. G. MCDONALD. 1988a. Blood gases, acid-base status, ions, and hematology in adult brook trout (*Salvelinus fontinalis*) under acid/aluminum exposure. Can. J. Fish. Aquat. Sci. 45: 1575–1586.

WOOD, C. M., D. G. MCDONALD, C. E. BOOTH, B. P. SIMONS, C. G. INGERSOLL, AND H. L. BERGMAN. 1988b. Physiological evidence of acclimation to acid/aluminum stress in adult brook trout (*Salvelinus fontinalis*). 1. Blood composition and net sodium fluxes. Can. J. Fish. Aquat. Sci. 45: 1587–1596.

WOOD, C. M., B. P. SIMONS, D. R. MOUNT, AND H. L. BERGMAN. 1988c. Physiological evidence of acclimation to acid/aluminum stress in adult brook trout (*Salvelinus fontinalis*). 2. Blood parameters by cannulation. Can. J. Fish. Aquat. Sci. 45: 1597–1605.

WOOD, C. M., D. G. MCDONALD, C. G. INGERSOLL, D. R. MOUNT, O. E. JOHANNSSON, S. LANDSBURGER, AND H. L. BERGMAN. 1990a. Effects of water acidity, calcium and aluminum on whole body ions of brook trout continuously exposed from fertilization to swim-up: A study by instrumental neutron activation analysis. Can. J. Fish. Aquat. Sci. 47: 1593–1603.

1990b. Whole body ions of brook trout alevins: responses of yolk-sac and swim-up stages to water acidity, calcium, and aluminum, and recovery effects. Can. J. Fish. Aquat. Sci. 47: 1614–1625

Effects of pH, Aluminum, and Calcium on Survival and Growth of Eggs and Fry of Brook Trout (*Salvelinus fontinalis*)

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Ingersoll, C: G., D. R. Mount, D. D. Gulley, T. W. La Point, and H. L. Bergman. Effects of pH, aluminum, and calcium on survival and growth of eggs and fry of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47:1580–1592.

Freshly fertilized eggs, eyed eggs, yolk-sac fry, and swim-up fry of brook trout (*Salvelinus fontinalis*) were exposed to a matrix of 84 combinations of pH (4.0–6.5), aluminum (0–1000 μg/L), and calcium (0.5–8.0 mg/L) in 21–91-d experiments. The response to pH, aluminum, and calcium exposure was dependent on life stage. Sensitivity to acid toxicity generally decreased with age (freshly fertilized eggs>eyed eggs>yolk-sac fry=swim-up fry). Survival or hatching of freshly fertilized and eyed eggs decreased at pH 5.2 and below, whereas survival of yolk-sac and swim-up fry was reduced at pH 4.4–4.0. Sensitivity to aluminum toxicity generally increased with age (freshly fertilized eggs=eyed eggs<yolk-sac fry<swim-up fry). At low pH, survival of freshly fertilized and eyed eggs increased with exposure to increasing aluminum concentrations. In contrast, elevated aluminum (≥333 μg/L)was often toxic to fry. Increasing calcium was beneficial to all life stages, although the magnitude of this benefit depended on life stage and on the specific pH and aluminum concentration. Survival after previous exposure to toxic combinations of pH, aluminum, and calcium generally improved during a post-exposure recovery period at pH 6.5. However, increased mortality in many exposure combinations did not begin until this recovery period.

Des oeufs fraîchement fécondés, des oeufs embryonnés, des larves vésiculées et des alevins d'omble de fontaine (*Salvelinus fontinalis*) ont été exposés à une matrice de 84 combinaisons de pH (4,0 à 6,5), de concentrations d'aluminium (0 à 1000 μg/L), et de calcium (0,5 à 8,0 mg/L) dans le cadre d'expériences d'une durée de 21 à 91 jours. La réaction à une exposition au pH, à des concentrations d'aluminium et de calcium dépendait du stade de développement atteint. La vulnérabilité à la toxicité acide diminuait en général avec l'âge (oeufs fraîchement fécondés>oeufs embryonnés>larve vésiculée = alevin). La survie ou l'éclosion des oeufs fraîchement fécondés et des oeufs embryonnés diminuait à pH 5,2 ou à pH inférieur, tandis que la survie des larves vésiculées et des alevins était réduite lorsque le pH était compris entre 4,4 et 4,0. La vulnérabilité à la toxicité de l'aluminium augmentait en général avec l'âge (oeufs fraîchement fécondés = oeufs embryonnés<larves vésiculées<alevins). À pH faible, la survie des oeufs fraîchement fécondés et des oeufs embryonnés augmentait lorsqu'ils étaient exposés à des concentrations accrues d'aluminium. Par contraste, une teneur élevée en aluminium (supérieure ou égale à 333 μg/L) était souvent toxique pour les alevins. Une quantité accrue de calcium était bénéfique à toutes les étapes de la vie bien que l'ampleur de cet avantage soit fonction du stade de développement, du pH spécifique et de la teneur en aluminium. La survie après une première exposition à des combinaisons toxiques de pH, d'aluminium et de calcium augmentait en général au cours d'une période de rétablissement à pH 6,5 après une exposition. Toutefois, l'augmentation de la mortalité dans le cas de nombreuses combinaisons d'exposition ne commençait pas à se manifester avant cette période de rétablissement.

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Acidification of surface waters in Scandinavia (Wright and Gjessing 1976), Ontario (Beamish and Harvey 1972) and the northeastern United States (Schofield 1976) has led to the loss of valuable fisheries (Baker 1984). However, acid stress is not the sole factor causing these declines. In a survey of lakes in southern Norway, Wright and Snekvik (1978) observed that fish abundance declines concomitantly with either decreasing calcium or decreasing pH. In addition, increased mobilization of aluminum into lakes and streams accompanies long-term acidification of most watersheds (Cronan and Schofield 1979; Driscoll 1980; Seip 1980; LaZerte 1984). Thus the interactions among aluminum, calcium, and pH must be considered when assessing the effects of acidification on the survival of fish populations in these low conductivity waters (e.g. Muniz and Leivestad 1980 a,b; Driscoll et al. 1980; Brown 1983; Sevaldrud and Skogheim 1986).

Recruitment failure is considered as a primary mechanism leading to the gradual loss of fish populations in acidified waters (Jensen and Snekvik 1972). How this recruitment failure occurs is unclear. The inability of female fish to release mature eggs may be a causal mechanism of recruitment failure (Beamish et al. 1975; Lockhart and Lutz 1977; Frennette and Dodson 1984). However, egg and fry mortality are considered the major cause of declining fish populations by many (Leivestad et al. 1976; Schofield 1976; Harvey 1979; Rosseland et al. 1980).

Numerous laboratory studies have tested the sensitivity of young fish to acidification (see Baker 1984; Marcus et al. 1986), but only Brown (1983) and Kostecki et al. (1985) investigated the combined effects of pH, aluminum, and calcium on early

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TABLE 1. Nominal pH levels and aluminum concentrations (μg/L) for the 91-d and age-specific experiments. Nominal calcium concentrations for each pH and aluminum combination were 0.5, 1.0, 2.0, and 8.0 mg/L.

pH	Aluminum (μg/L)				
6.5	0	—	—	—	—
5.2	0	12	37	111	333
4.8	0	12	37	111	333
4.4	0	37	111	333	1000
4.0	0	37	111	333	1000

life stages of fish. Several studies have compared the sensitivity of fish to acidification during more than one life stage (Mount 1973; Menendez 1976; Craig and Baksi 1977; Swarts et al. 1978; Lee and Gerking 1980; Baker and Schofield 1982; Jagoe et al. 1984; Rask 1984; Kwain and Rose 1985; Cleveland et al. 1986; Hunn et al. 1987).

The objectives of the present study were to: (1) determine the long-term response of brook trout continuously exposed for 91 d from the freshly fertilized egg life stage through the swim-up fry life stage to pH, aluminum, and calcium levels representative of acid-sensitive natural waters, (2) compare the relative response of four individual early life stages (freshly fertilized eggs, eyed eggs, yolk-sac fry, and swim-up fry) exposed for 21–28 d to the response of the fish exposed continuously for 91 d from the freshly fertilized egg stage until the swim-up fry stage; and (3) determine the ability of fish exposed in each of these four individual life stage experiments to recover from previous exposure to toxic combinations of pH, aluminum, and calcium. Histological and physiological responses of early life stage brook trout to pH, aluminum, and calcium exposure are discussed in the accompanying series of papers on histopathology (Ingersoll et al. 1990a) and ionoregulation (Wood et al. 1990 a,b). Additional papers in this issue (Bergman et al. 1990) and in a previous issue of this journal (Bergman et al. 1988) present additional toxicological, physiological, and histopathology results for studies with early life stage, juvenile, and adult brook trout exposed in the laboratory to water conditions typical of acidified lakes and streams.

Materials and Methods

Exposure Conditions

Eighty-four combinations of pH, aluminum, and calcium were delivered simultaneously to duplicate exposure chambers in a flow-through system (Table 1). Well water (250 mg/L hardness as $CaCO_3$, pH 7.8) treated by reverse osmosis and deionization (Ingersoll 1986) was delivered to an 800-L head tank where 1 mg Na/L was added as NaCl. The pH of this water was adjusted to pH 6.5 with a pH controller (Leeds and Northrup Model 7083) using either dilute H_2SO_4 or NaOH. This pH-adjusted water was chilled and mixed by a 1-hp chiller (Frigid Units Model D1-100, Toledo, OH) and then delivered to five head tanks where the pH was lowered in four of these head tanks (again using dilute H_2SO_4 and pH controllers). Circulating pumps (Model AC-2CP-MD, March Mfg. Inc., Glenview, IL) vigorously mixed and aerated the water in these tanks. The pH-adjusted water from each of these four head tanks was split four ways with capillary tubing to four separate proportional dilution systems (modified from Mount and Brungs 1967). Mariotte bottles metered calcium (as $CaCl_2$) into the pH-adjusted dilution water at each diluter. Aluminum was added (as $AlCl_3$) via a toxicant cell with each cycling of the diluter. Each unique combination of pH- , calcium- , and aluminum-adjusted water was split and delivered to duplicate 600-mL exposure chambers that received 300 mL/cycle and 65 volume additions daily. At pH 6.5, aluminum was not an experimental variable. At this pH, four levels of calcium were maintained (in triplicate) with a single proportional diluter delivering calcium via a toxicant cell. Thus, for the 84 combinations of pH, aluminum, and calcium, 17 diluters were maintained: four at each of the four pH levels below pH 6.5, where calcium and aluminum were added, and one at pH 6.5 where only calcium was added.

Exposure Procedures

Twenty-five freshly fertilized or eyed eggs were placed into each egg cup at the start of each experiment initiated with eggs. These egg cups (7-cm glass cylinder, 3.5-cm I.D., 6 mesh/cm Nitex^R screen) were suspended in exposure chambers and raised and lowered about 2 cm through the exposure water during each 30-s cycle. For experiments starting with yolk-sac or swim-up fry, 25 fish were placed directly into each exposure chamber at the beginning of each experiment. Daily observations were recorded for survival or hatching. Newly hatched fry, once free from the egg membrane (chorion), were removed from the egg cups and placed into the exposure chambers. At swim-up, fry were fed Rangens Trout Starter (Rangens Inc., Buhl, ID) at about 4% of their body weight per day, and exposure chambers were cleaned daily. For eggs, death was defined as the appearance of any opaque material within the chorion; for fry, death was defined as the lack of visible movement with the unaided eye after gentle prodding with a blunt probe; death while hatching was determined by recording the number of fry that died after the chorion had ruptured but before the fish were free from the chorion; hatching time was calculated as the mean for each replicate exposure chamber. At the end of most of the experiments, wet weight of individual fish was measured to the nearest 0.1 milligram. Photoperiod was 12-h light/12-h darkness. Temperature averaged 10.9°C (range 8.7–12.0°C; Standard error of the mean SEM = 0.04) in all of the experiments. Temperature was maintained by adjusting the delivery of cold water (~7°C) in a circulating water bath surrounding the exposure chambers.

Experiments

91-d freshly fertilized egg experiment

On 17 December 1984, 26 000 freshly fertilized eggs were obtained from Beity's Resort, Valley, WA (water hardness100 mg/L as $CaCO_3$). At the hatchery, about 40 male and 40 female brook trout were spawned into soft water (pH 6.5, 2 mg Ca/L, 1 mg Na/L (nominal), at ~4°C). Five minutes after spawning, these eggs were rinsed four times with this soft water, and within 1 h of spawning, four subsets of these eggs were placed into separate containers of pH-adjusted water (initially, pH 5.2, 4.8, 4.4, and 4.0, each at 2 mg Ca/L, 1 mg Na/L (nominal)). During air transport to our laboratory in Laramie, the pH of each container was adjusted, every 2 h if necessary, with H_2SO_4 (Ingersoll 1986). The eggs arrived at the laboratory with 8 h of fertilization and, within the next 2 h, were transferred into each of the appropriate combinations of aluminum and calcium (Table 1). The levels of pH, aluminum, and calcium were chosen to represent the chemistry of sensitive lakes and streams in

Scandinavia, eastern Canada, and the northeastern United States. Fish were exposed to higher aluminum concentrations at lower pH levels because dissolved inorganic aluminum concentration increases as pH decreases in acidified surface water (e.g. Schofield 1982). This experiment was conducted for 91 d from fertilization through the swim-up life stage.

Age-specific exposure and recovery experiments

Four separate life stages were exposed to the same 84 combinations of pH, aluminum, and calcium (Table 1) concurrently with the 91-d freshly fertilized egg experiment: (1) freshly fertilized eggs, exposed from fertilization through day 28 of development (to the eyed egg stage); (2) eyed eggs, exposed for 21 d from the eyed stage until the initiation of hatching; (3) yolk-sac fry, exposed for 21 d from hatching until the swim-up fry life stage; and (4) swim-up fry, exposed for 21 d starting at swim-up. The sum of these four exposures is equivalent to the 91-d exposure described above. After each exposure, surviving fish from each of these age-specific experiments were transferred directly to water adjusted to pH 6.5, 2.0 mg Ca/L, and 1.0 mg Na/L (nominal), and survival was monitored during a recovery period lasting 20–21 d. The fish in each of the four age-specific experiments were exposed in the same chambers used in the 91-d freshly fertilized egg experiment. The exposure chambers were subdivided into two separate areas with Nitex^{R} screen (6 mesh/cm). The back of the exposure chamber held fish for the continuous 91-d experiment, and the front of the chamber held fish for the age-specific experiments (without replication). Eggs not used in the 91-d experiment were held at pH 6.5, 2.0 mg Ca/L, 1.0 mg Na/L (nominal), and 10°C until the start of eyed egg, yolk-sac fry and swim-up fry exposures.

Water Chemistry

The flow rate of calcium stock solution from each Mariotte bottle and the pH (Orion Ross combination pH electrode) of the water delivered to each diluter was measured daily and adjusted when necessary. The pH of the exposure chambers was measured and recorded at least weekly. Calcium, sodium, potassium, and aluminum were measured with a Perkin–Elmer (Model 2380) atomic absorption spectrophotometer. Aluminum was fractionated into total (TA1) and monomeric (MA1; fast-reactive) after the method of LaZerte (1984). Chloride, fluoride, and sulfate were measured with a Dionex (Model 2110i and a #AS-40 column) automated single-channel ion chromatograph. Dissolved oxygen (YSI Oxygen meter Model 54A) and total ammonia (Orion Research Ionoalyzer Model 407A) were routinely measured in all experiments.

Nominal and measured pH agreed closely, but measured calcium averaged about 25% less than nominal concentrations (Table 2). Measured total aluminum (TA1) and monomeric aluminum (MA1) were in close agreement with nominal aluminum concentrations in all of the experiments (Table 2). Subsequent chemical analyses at our laboratory using the same exposure system (Parkhurst 1987) indicated that this monomeric aluminum was mainly inorganic monomeric aluminum (LaZerte 1984).

All statistical analyses for the 91-d and 21-d eyed egg, yolk-sac fry, and swim-up fry experiments were based on monomeric aluminum concentrations. In the 28-d freshly fertilized egg experiment monomeric aluminum was not determined, so statistical analyses were based on measured total aluminum concentrations. Statistical analyses in the age-specific experiments were based on measured chemistry during each exposure period. These values were in close agreement with measured chemistry in the 91-d experiment. For simplicity, we refer to the measured concentrations from the 91-d experiment when discussing exposure water chemistry for each of the age-specific experiments (Table 2).

TABLE 2. Nominal and measured values of pH, aluminum, calcium, sodium, chloride, sulfate, and fluoride in the 91-d and age-specific experiments.

	Measured values		
Nominal values	Mean	SD	*n*
pH			
4.0	4.0	0.09	242
4.4	4.4	0.08	275
4.8	4.8	0.11	275
5.2	5.2	0.16	282
6.5	6.5	0.17	96
Total aluminum (μg TAl/L)[b]			
0	6	6	76
12	20	7	32
37	54	16	60
111	126	24	60
333	350	21	60
1000	1090	138	28
Monomeric aluminum (μg MAl/L)[b]			
0	5	6	76
12	18	12	23
37	48	15	36
111	121	25	36
333	349	37	34
1000	1167	204	9
Calcium (mg/L)[c]			
0.5	0.37	0.1	74
1.0	0.75	0.2	71
2.0	1.48	0.8	84
8.0	6.23	1.1	84
Sodium (mg/L)[d]	3.8	1.0	315
Chloride (mg/L)[d]	8.0	5.7	316
Sulfate (mg/L)[d]	8.0	3.8	313
Fluoride (mg/L)[d]	0.023	0.016	313

[a] mean calculated across aluminum and calcium.

[b] mean calculated across pH and calcium.

[c] mean calculated across pH and aluminum.

[d] mean calculated across pH, aluminum, and calcium.

The pH of the exposure chambers nominally at pH 4.8 and 5.2 and high aluminum concentrations (111–333 μg/L) was consistently 0.05–0.10 pH units lower than the exposure chambers at pH 4.8 and 5.2 without aluminum addition. The pH of the aluminum stock solution (about pH 4.0) lowered the pH in these chambers. Dissolved oxygen was greater than 88% saturation (range 7.2–8.7 mg/L), and total ammonia was less than 0.15 mg/L in all experiments.

Statistics

Partial correlation analyses (Sokal and Rohlf 1981) were used to determine linear effects of pH (independent of calcium and aluminum), calcium (independent of pH and aluminum), and aluminum (independent of pH and calcium) on fish survival, weight, and hatching at significance levels of alpha = 0.05.

Survival data were analyzed with stepwise logistic regression. Proportion surviving was assumed to fit a logistic distribution.

$$E(y) = \frac{\exp(\beta_0 + \beta_1 X_1 \ldots + \beta_n X_n)}{1 + \exp(\beta_0 + \beta_1 X_1 \ldots + \beta_n X_n)}$$

where β_0–β_n are the parameters of the regression model, X_1 to X_n are the independent variables, and y is the proportion surviving. Before the logistic regression analyses were performed, percent survival was normalized to the highest survival across the exposure matrix for each experiment. Linear stepwise regression was performed on data for weight and hatching time to predict the effects of pH, aluminum, and calcium on these two variables. All dependent variables were regressed against both non-transformed and $\log_{10}$-transformed chemistry values, except for acidity which was always expressed as pH. Our goal in the regression analyses was to define simple models that would identify the key variables relating to each measured response. For this reason we limited the models to first-order terms for pH, aluminum, calcium, and the interactions between these variables. In addition to these first-order terms, in some of the models we included a second-order aluminum term to fit the non-linear response of the aluminum effect. For logistic regressions, variables were included if they significantly improved (alpha = 0.05) the goodness of fit Chi-square. For linear regressions, the F statistic was used to determine the significance of the regression (alpha = 0.05), and only variables that increased the adjusted R^2 of the model were considered for inclusion in the final model (Mount et al. 1988).

Three-dimensional response surfaces were plotted using the regression models and Golden Graphics Software (Golden, Colorado). At pH 6.5, aluminum was not an experimental variable, so these data were not included in either the regression models or the response surfaces. Aluminum was not tested above 333 μg/L at pH 4.8 or pH 5.2, (Table 1), so the values predicted by the models at these exposure combinations were not included in the response surface plots. In addition, survival data were plotted as three-dimensional bar graphs using Golden Graphics Software. This allowed a direct comparison of the predicted response surface by the regression models to a three-dimensional representation of the actual data. Only selected plots from either the actual data or response surfaces predicted from regression models were included in the Results section. For a complete listing of these data see Ingersoll (1986). In addition, the raw data are available on computer disc or tape from the Electric Power Software Center (Bergman et al. 1988).

Stepwise logistic regressions were performed with the BMDPLR software package (Dixon et al. 1985). The partial correlations, ANOVAs, and linear stepwise regressions were performed with SPSS (Nie et al. 1975, Neter et al. 1985). All statistical analyses were performed with the University of Wyoming's mainframe computers (Control Data Corp., Cyber 840).

Results

91-D Freshly Fertilized Egg Experiment

Survival of fish held at pH 6.5, 6.23 mg Ca/L was only 40%, yet survival of fry after hatching was greater than 96%. Fertilization may have been less than 50% at the start of the 91-d experiment. A separate experiment was therefore conducted to address this fertilization question. Non-fertilized eggs were held at pH 6.5 and after 32 d, 78% of these eggs had not turned opaque (our criterion for death). This suggests the low survival to hatching in the 91-d experiment could have been the result of starting the experiment with a high percentage of eggs that were not fertilized.

Survival of eggs and resultant fry in the 91-d exposure period was affected positively (e.g. significant positive correlation) by calcium and negatively (e.g. significantly negative correlation) by acidity (Fig. 1). Fry survival after hatching increased with increasing calcium and decreased with increasing aluminum.

Comparisons of the 91-d survival data (Fig. 2) to the response predicted from the regression model (Fig. 3) indicate that the regression model adequately describe the actual data. The only discernible inconsistency occurred at pH 5.2, 1.48 mg Ca/L and 349 μg MA1/L where the model predicted survival of about 80% and the actual survival was about 40%. While the bar graphs of the actual survival data in Fig. 2 can be used to illustrate general trends, interpretations across the entire exposure matrix are most clearly made comparing the response surfaces generated by the model (Fig. 3).

The response surfaces predicted from the 91-d survival model were strongly dependent on pH, aluminum, and calcium (Fig. 3). A 50% reduction in survival was predicted with exposure to pH 5.2, 5 μg MA1/L and 0.37 mg Ca/L. At the lower calcium concentrations, no fish survived either pH ≤4.4 or 1167 μg MA1/L and survival generally increased across the entire exposure matrix with increasing calcium concentrations (Fig. 2, 3). Effects of exposure to low calcium were also evident at pH 6.5 where survival was reduced at calcium concentrations below 6.23 mg/L (Fig. 2). Aluminum generally improved survival at intermediate exposure concentrations of about 121–349 μg MA1/L, which explains the non-significant linear correlation of aluminum to 91-d survival (Fig. 1).

Fry weight at the end of the 91-d experiment was reduced with increasing acidity and increased with increasing calcium or aluminum (Fig. 1). The predicted response surface was most strongly influenced by pH and calcium (Fig. 4). Predicted weight of surviving fry exposed to pH ≤5.2 and 0.37 mg Ca/L was reduced by about 50% relative to controls (pH 6.5, 6.23 mg Ca/L, and 5 μg MA1/L). Increasing calcium to 6.23 mg/L at pH ≤5.2 still resulted in about a 30% reduction in weight.

Neither survival during hatching nor percent hatching were affected by calcium or aluminum (Fig. 1). However, the percent fry hatching decreased with increasing acidity. While increasing acidity, calcium, or aluminum exposure delayed hatching time (Fig. 1), the delay was generally less than 5 d relative to controls (mean hatching time of 47.5 d at pH 6.5, 6.23 mg Ca/L, 5 μg MA1/L; Ingersoll 1986).

Age-Specific Exposure and Recovery

Survival

The response of brook trout to pH, aluminum, and calcium exposure was strongly dependent on life stage. Again, comparisons of the actual data (Fig. 5) to the modeled data (Fig. 6) indicate that the regression models adequately describe the survival data for each life stage.

1) Freshly fertilized egg experiment

Survival of freshly fertilized eggs in the 28-d experiment increased with increasing calcium and decreased with increasing acidity (Fig. 7). The response of freshly fertilized eggs to acid and aluminum exposure in the 28-d experiment was similar

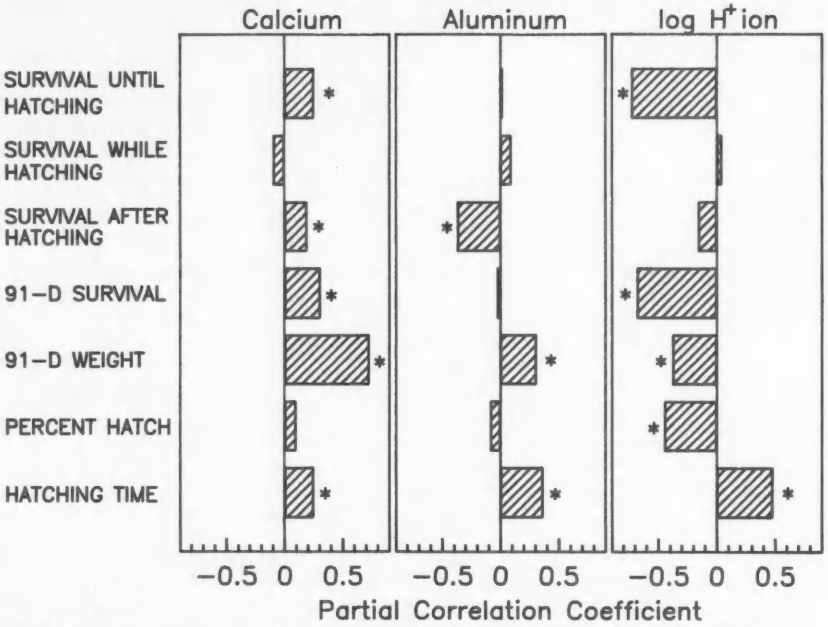

FIG. 1. Partial correlation coefficients between response variables measured and the independent variables calcium, aluminum, and log [H$^+$] ion in the 91-d freshly fertilized egg experiment.($* = p \leqslant 0.05$).

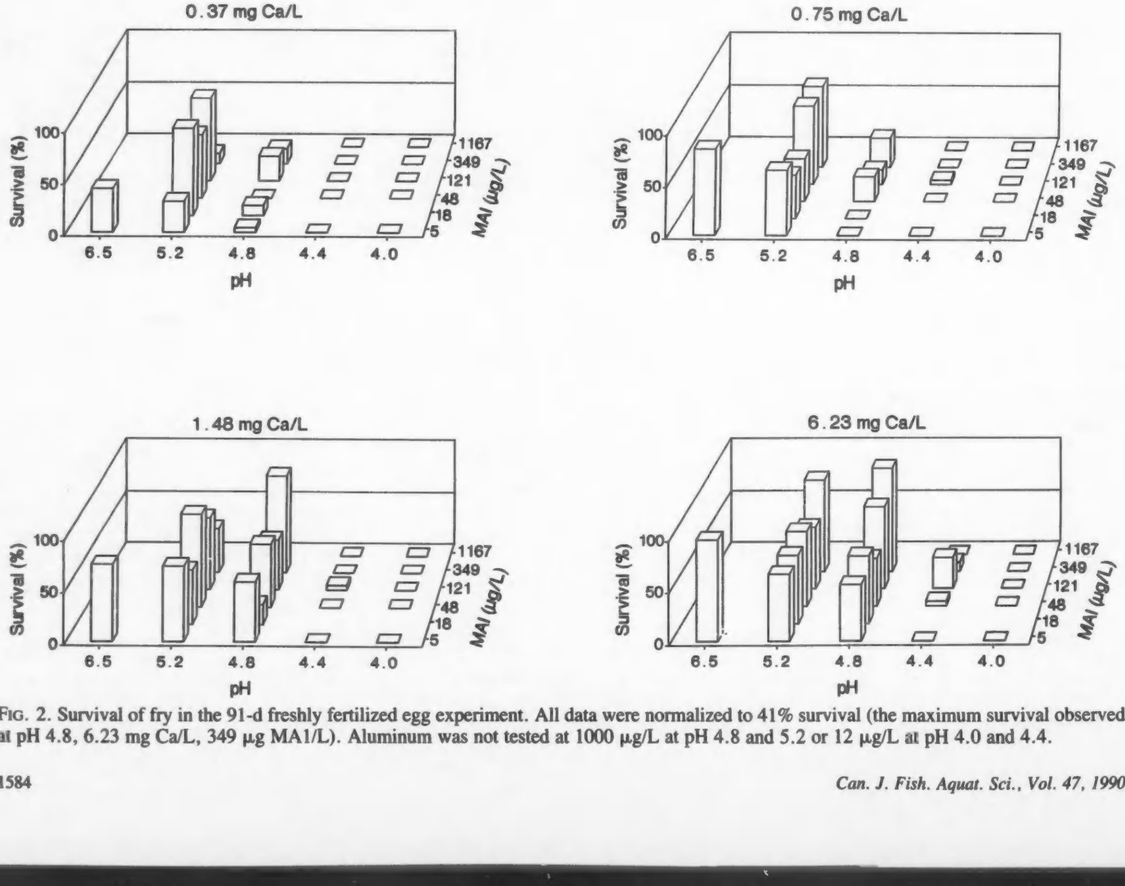

FIG. 2. Survival of fry in the 91-d freshly fertilized egg experiment. All data were normalized to 41% survival (the maximum survival observed at pH 4.8, 6.23 mg Ca/L, 349 µg MA1/L). Aluminum was not tested at 1000 µg/L at pH 4.8 and 5.2 or 12 µg/L at pH 4.0 and 4.4.

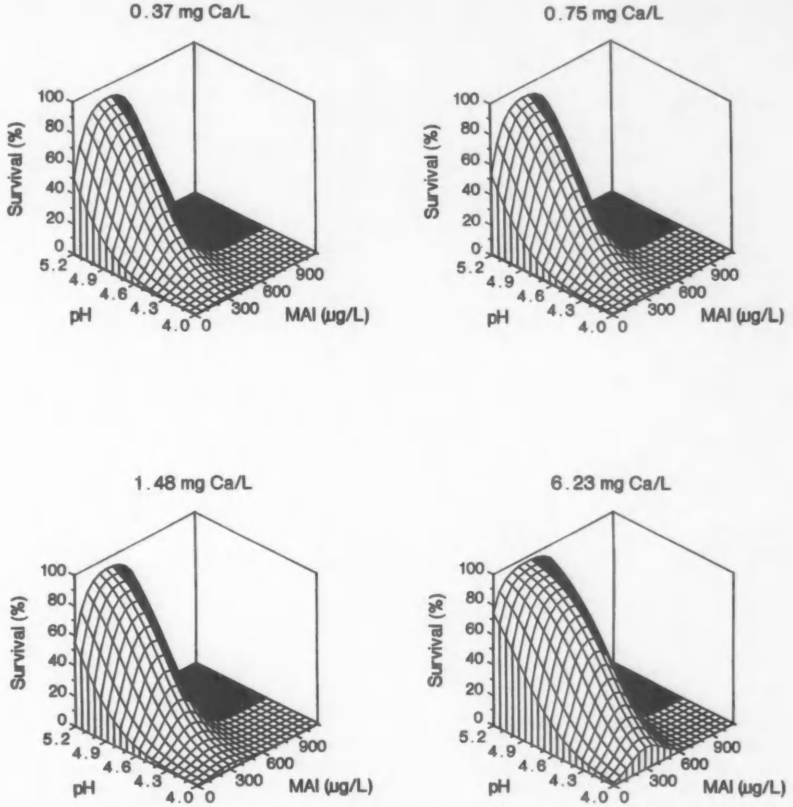

FIG. 3. Survival in the 91-d freshly fertilized egg experiment predicted by the fitted regression model: Logit survival $= 5.15$ pH $+ 1.13 \times 10^{-3}$ MAl $- 2.51 \times 10^{-5}$ MAl^2 $+ 9.42 \times 10^{-1}$ Ca $- 1.47 \times 10^{-1}$ pH*Ca $+ 4.77 \times 10^{-4}$ pH MAl $- 26.9$ ($R^2 = 0.76$). All data were normalized to 41% survival (the maximum survival observed at pH 4.8, 6.23 mg Ca/L, 349 µg MAl/L). The response predicted in the darkened area should not be interpreted since these combinations of pH, calcium, and aluminum were not tested.

to the full 91-d exposure. A 40% reduction in survival was predicted at pH ≤5.2 and 6 µg TAl/L (Fig. 6a). Aluminum generally improved survival at intermediate exposure concentrations of about 54–350 µg TAl/L (Fig. 5a), which explains the non-significant linear correlation of aluminum to freshly fertilized egg survival (Fig. 7). Increasing calcium (plots not included; Ingersoll 1986) generally increased survival.

Survival during the 21-d recovery period in pH 6.5 water increased relative to survival during the previous 28-d exposure to combinations of pH, aluminum, and calcium (Ingersoll 1986). Yet, survival during recovery was negatively correlated with previous aluminum exposure during the freshly fertilized egg stage (Fig. 7; see Ingersoll (1986) for actual data).

2) Eyed egg experiment

Eyed egg survival increased with both increased calcium and aluminum, and decreased with increased acidity (Fig. 7). Predicted survival was decreased by >20% at pH ≤5.2 and 5 µg MAl/L (Fig. 6b). However, survival generally increased with exposure to increasing aluminum concentrations (Fig. 5b, 6b). While survival of the eyed eggs in the 21-d experiment was reduced by >20% at pH 5.2, this life stage was fairly tolerant of lower pH. Survival at pH 4.0 was about 40–50% in the 21-d eyed egg experiment (Fig. 5b, 6b), whereas survival in the other life stages at pH 4.0 was <10% (Fig. 5, 6).

At the end of the 21-d exposure, fish held at pH ≤5.2 had not hatched and the chorion had not begun to break. However, most of these fish began to hatch within 48-h of being transferred to pH 6.5 recovery water. The survival of fish previously exposed to toxic conditions as eyed eggs during the 21-d exposure generally improved during the 20-d recovery period, but survival during this recovery was negatively correlated with previous exposure to low pH (Fig. 7; Ingersoll 1986).

3) Yolk-sac fry experiment

Survival of yolk-sac fry during the 21-d exposure period was reduced by either increasing aluminum or increasing acidity (Fig. 7). Yolk-sac fry were relatively tolerant of acid stress. Survival was reduced only at pH 4.0–4.4 at 5 µg MAl/L (Fig. 5c, 6c). Aluminum was only toxic to fry at 1167 µg MAl/L (Fig. 5c, 6c) and calcium had little influence on survival (plots not included). Previous exposure to acidity during the yolk-sac fry stage decreased survival during recovery (Fig. 7). None of the fry surviving the 21-d exposure to pH 4.0 lived through the 20-d recovery period. However, survival after exposure to pH 4.4 or higher was not reduced during recovery relative to

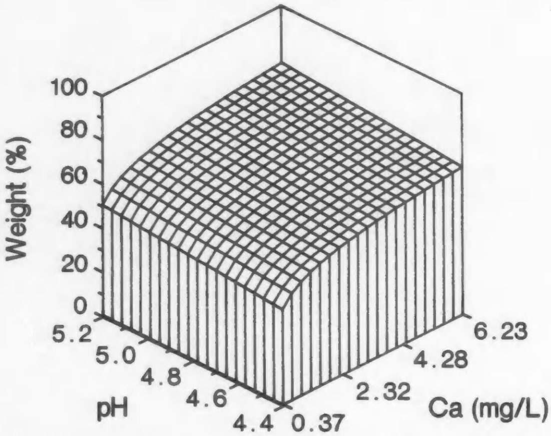

FIG. 4. Fry weight in the 91-d freshly fertilized egg experiment with exposure to 5 μg MA1/L predicted by the fitted regression model: Weight (mg) = 6.40 pH + 4.87 log(MA1) + 14.2 log(Ca) + 6.51 ($R^2 = 0.68$). All data were normalized to 74.1 mg (the maximum weight observed at pH 6.5, 6.23 mg Ca/L, 5 μg MA1/L.

controls (pH 6.5, 6.23 mg Ca/L and 5 μg MA1/L; Ingersoll 1986).

4) Swim-up fry experiment

As in the yolk-sac fry experiment, survival of swim-up fry was reduced with either increasing aluminum or increasing acidity (Fig. 7). Swim-up fry were also relatively tolerant of acid stress. Survival was reduced at pH 4.0–4.4 at 5 μg MA1/L (Fig. 5d, 6d). Aluminum toxicity increased from the yolk-sac fry to the swim-up fry life stage. Swim-up fry survival was generally lower at 349–1167 μg MA1/L (Fig. 6d) compared to yolk-sac fry survival at these exposure combinations (Fig. 6c). While calcium had only a minor influence on survival of swim-up fry during the initial 21-d exposure (response surfaces not included), previous exposure to elevated calcium increased survival during the 20-d recovery (Fig. 7). Swim-up fry from many of the exposure combinations did not begin to die until this recovery period (e.g. Fig. 8). As seen in the eyed egg and yolk-sac fry experiments, previous exposure to low pH decreased survival of the swim-up fry during the 20-d recovery period (Fig. 7; Ingersoll 1986).

Weight

Fry weight was measured at the end of the 21-d yolk-sac and swim-up fry exposures and at the end of the eyed egg, yolk-sac fry, and swim-up fry recovery periods. Only in the 21-d swim-up fry experiment was weight significantly affected by exposure conditions. Swim-up fry weight in the 21-d experiment was positively correlated with calcium ($r = 0.72$, $p \leq 0.05$) and negatively correlated with acidity ($r = -0.66$, $p \leq 0.05$). As was observed in 91-d experiment, the predicted response surface for swim-up fry weight was dependent on pH and calcium exposure (Fig. 9). Predicted weight of surviving swim-up fry exposed to pH 4.0 and 0.37 mg Ca/L was about 50% less than controls. Increasing exposure pH to 5.2 at 0.37 mg Ca/L or increasing calcium to 6.23 mg/L at pH 4.0 still resulted in about a 30% reduction in weight. Only with exposure to pH 5.2 and 6.23 mg Ca/L was predicted weight of surviving fry not reduced relative to control fish.

Discussion

The response of brook trout to combinations of pH, aluminum, and calcium varied among life stages. Sensitivity to acid exposure generally decreased with age (freshly fertilized eggs>eyed eggs>yolk-sac fry = swim-up fry). Survival of freshly fertilized and eyed eggs was reduced at pH levels below 5.2, whereas survival of yolk-sac and swim-up fry did not decline until about pH 4.0–pH 4.4. A decrease in sensitivity to acid with increasing age was also apparent in the 91-d freshly fertilized egg experiment and in a 40-d experiment initiated with eyed eggs (Ingersoll et al. 1985; Ingersoll 1986). In both the 91-d and 40-d experiments, egg survival or hatching were

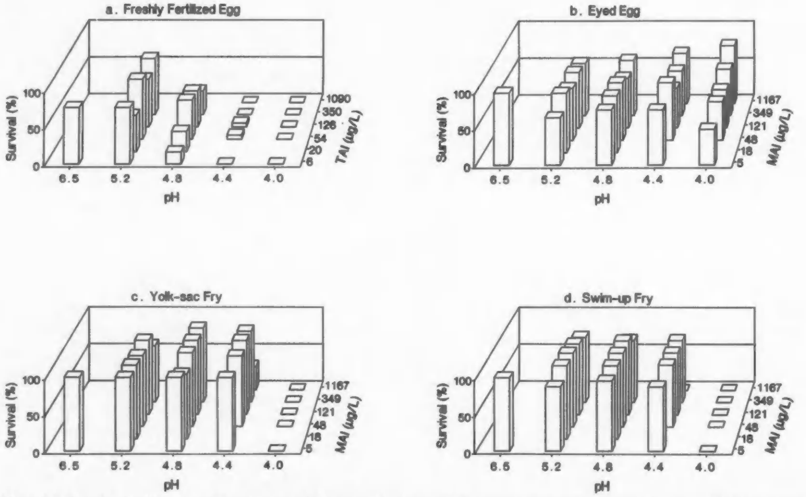

FIG. 5. Survival of fry in the age-specific experiments with exposure to 1.48 mg Ca/L. All data were normalized to highest survival during each exposure (see Fig. 6). Aluminum was not tested at 1000 μg/L at pH 4.8 and 5.2 or 12 μg/L at pH 4.0 and 4.4.

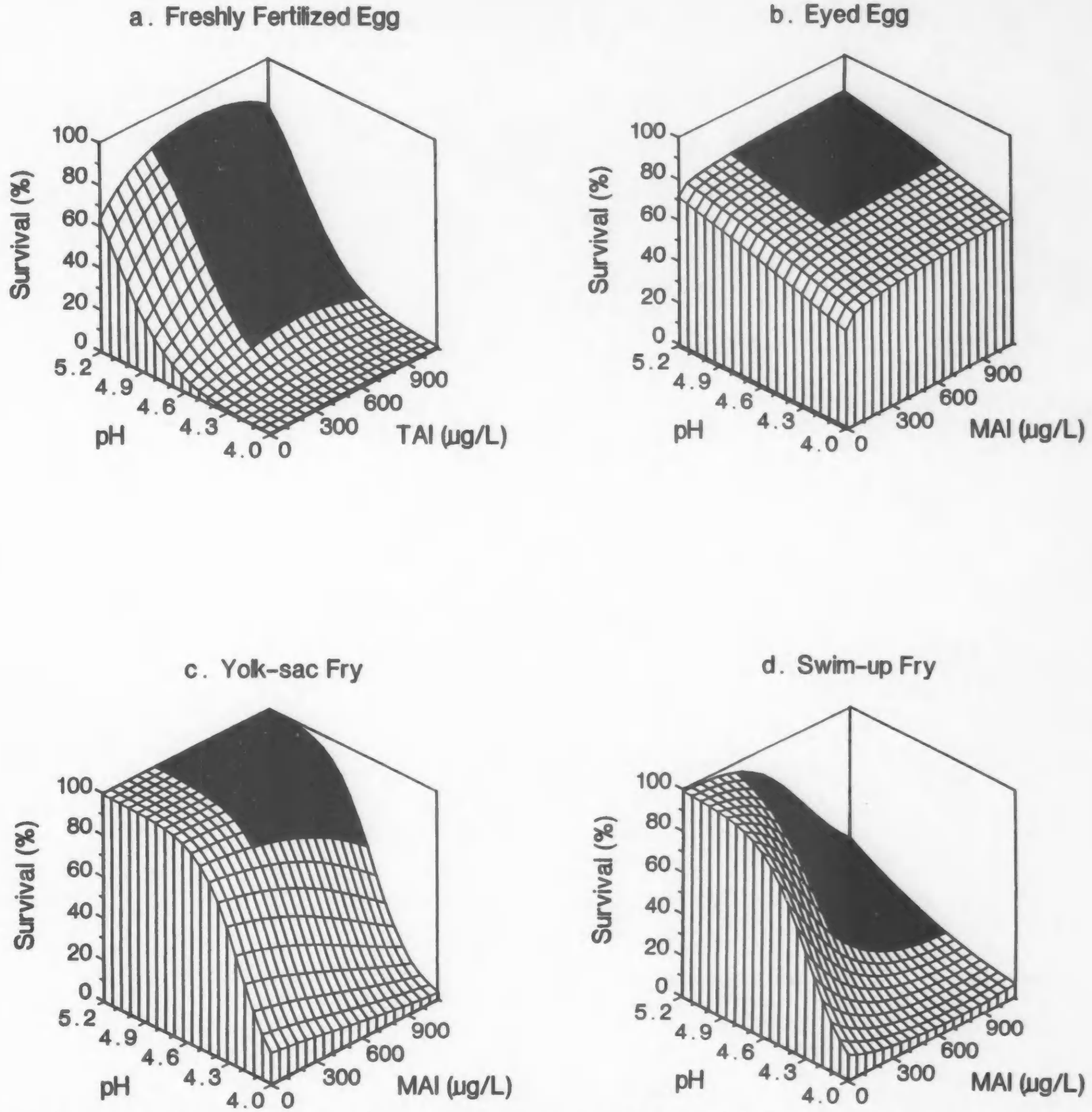

FIG. 6. Survival in the four age-specific experiments predicted by the fitted regression models with exposure to 1.48 mg Ca/L. All data were normalized to highest survival during each exposure. The response predicted in the darkened area should not be interpreted since these combinations of pH, calcium, and aluminum were not tested. (a) *28-d freshly fertilized egg experiment*: Logit survival $= 6.05\ \mathrm{pH} + 5.25 \times 10^{-3}\ \mathrm{TA1} - 3.61 \times 10^{-6}\ \mathrm{TA1}^2 + 2.54\ \mathrm{Ca} - 4.64 \times 10^{-1}\ \mathrm{pH^*Ca} - 4.57 \times 10^{-4}\ \mathrm{TA1^*Ca} - 3.11$ ($R^2 = 0.79$; survival at pH 6.5, 6.23 mg Ca/L, 6 µg TA1/L = 73%); (b) *21-d eyed egg experiment*: Logit survival $= 1.05\ \mathrm{pH} + 2.36 \times 10^{-1} \log(\mathrm{MA1}) + 4.64 \log(\mathrm{Ca}) - 8.93 \times 10^{-1}\ \mathrm{pH^*Log(Ca)} - 4.64$ ($R^2 = 0.48$; survival at pH 6.5, 6.23 mg Ca/L, 5 µg MA1/L = 91%); (c) *21-d yolk-sac fry experiment*: Logit survival $= 7.08\ \mathrm{pH} - 7.08 \times 10^{-4}\ \mathrm{Al} - 8.41 \times 10^{-7}\ \mathrm{MA1}^2 - 9.69 \times 10^{-1}\ \mathrm{Ca} + 2.83 \times 10^{-1}\ \mathrm{pH^*Ca} - 30.3$ ($R^2 = 0.82$; survival at pH 6.5, 1.48 mg Ca/L, 5 µg MA1/L = 100%); (d) *21-d swim-up fry experiment*: Logit survival $= 4.91\ \mathrm{pH} + 1.41 \times 10^{-2}\ \mathrm{MA1} + 2.57 \times 10^{-6}\ \mathrm{MA1}^2 - 4.13\ \mathrm{Ca} + 1.02\ \mathrm{pH^*Ca} - 4.23 \times 10^{-3}\ \mathrm{pH^*MA1} - 4.68 \times 10^{-4}\ \mathrm{MA1^*Ca} - 21.5$ ($R^2 = 0.75$; survival at pH 6.5, 6.23 mg Ca/L, 5 µg MA1/L = 100%).

severely affected by low pH, yet once fry successfully hatched, survival was generally not reduced further by exposure to low pH. Moreover, this change in acid sensitivity occurred at the same life stage. Eyed eggs at the start of the 21-d experiment were at an earlier developmental stage than the eyed eggs at the start the 40-d experiment (Ingersoll et al. 1985) and as a result were more susceptible to the effects of pH, aluminum, and calcium exposure. Decreased sensitivity to acid with increasing age has been reported for early life stages of white sucker and brook trout (Trojnar 1977 a, b; Swarts et al. 1978; Baker and Schofield 1982; Kwain and Rose 1985). However, yolk-sac fry of Atlantic salmon *Salmo salar* (Daye and Garside

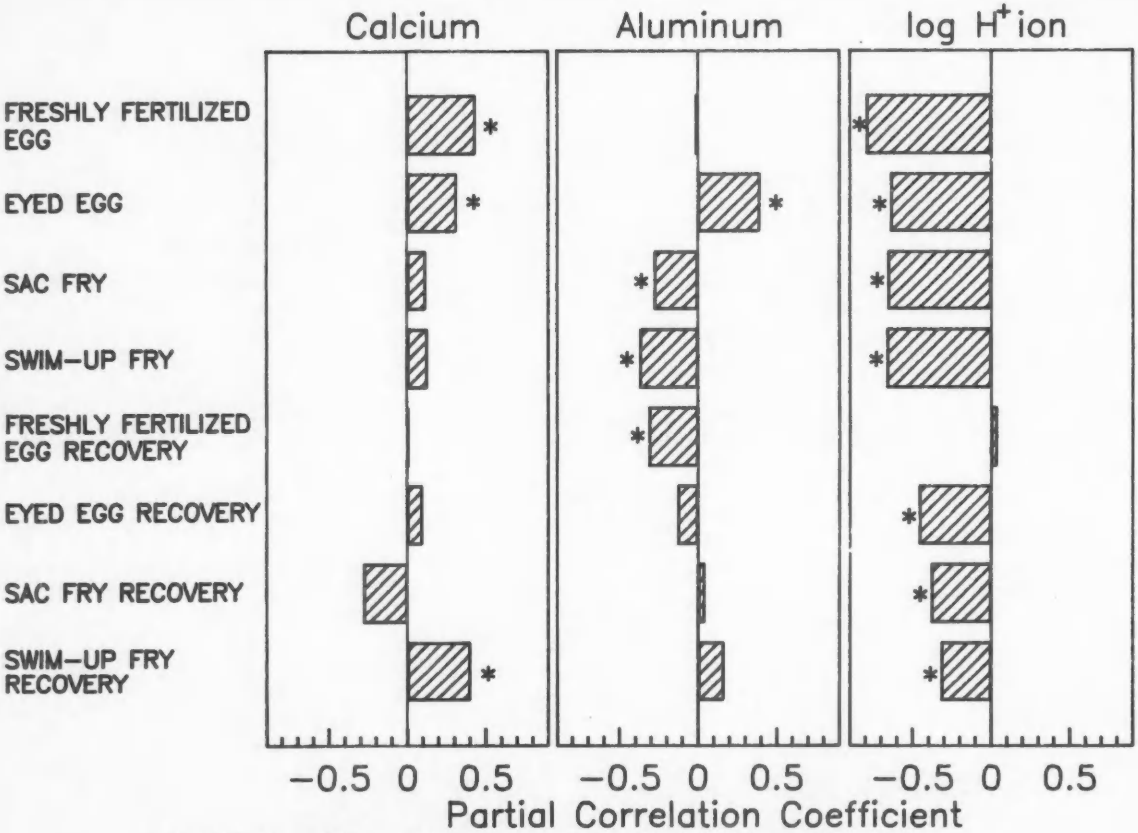

FIG. 7. Partial correlation coefficients between survival and the independent variables calcium, aluminum and log [H+] ion in the age-specific experiments. (* = $p \leq 0.05$).

1977, 1979; Daye 1980) perch *Perca fluviatilis*, (Rask 1984) and Pacific salmon (Rombough 1982) are extremely sensitive to acid stress compared to eyed eggs or swim-up fry.

In contrast to pH, sensitivity to aluminum exposure generally increased with age (freshly fertilized eggs = eyed eggs<yolk-sac fry<swim-up fry). Survival of freshly fertilized and eyed eggs generally increased with increasing aluminum exposure. However, aluminum concentrations of about 349–1167 μg MA1/L were often toxic to yolk-sac fry and swim-up fry. This aluminum-mediated decrease in acid toxicity to eggs may be due to polyvalent aluminum functioning similarly to calcium in controlling membrane permeability and ion transport at low pH (Schofield and Trojnar 1980). While protective effects of aluminum are often observed in the laboratory (e.g. Brown 1981; Baker and Schofield 1982; Dalziel et al. 1986), this ameliorative effect has not been demonstrated in the field (B. R. Parkhurst, Western Aquatics, Laramie, WY, pers. comm.). The increase in aluminum toxicity with age observed here and elsewhere (Baker and Schofield 1982; Brown 1983, Rosseland and Skogheim 1984; Cleveland et al. 1986; Hunn et al. 1987) may result from damaging of developing gills by aluminum in older fish (Hutchinson and Holtze 1985). A given concentration of aluminum is often more toxic at higher pH (e.g. Schofield and Trojnar 1980; Fivelstad and Leivestad 1984; Ingersoll et al. 1990b). While aluminum toxicity in our study sometimes increased with increasing pH, aluminum at lower pH was generally more toxic (Ingersoll 1986).

Our results on acid toxicity to brook trout swim-up fry agreed with those of Parkhurst et al. (1990), who used the same exposure system. However, sensitivity to aluminum differed. For example, Parkhurst et al. (1990) reported 21-d survival of 30–57% for swim-up fry at pH 5.2, 111 μg Al/L and 2 mg Ca/L (nominal), whereas swim-up fry survival was 96% at these exposure levels in our study (Fig. 5d). This difference in sensitivity to aluminum is probably the result of either age or strain of brook trout tested. Parkhurst et al. (1990) initiated exposures with older fish which were probably more sensitive to aluminum. Our experiments were conducted with a western brook trout strain (Beity's Resort, Valley, WA) and Parkhurst et al. (1990) tested a strain (Temiscamie-domestic hybrid) from New York. In subsequent experiments comparing these two strains, one year-old fish exhibited similar survival, growth and gill histological responses to aluminum and acid stress (Ingersoll et al. 1990b). This suggests the difference in aluminum sensitivity observed by Parkhurst et al. (1990) may have been due to a difference in age of the fish tested.

Increasing calcium concentration was beneficial to all life stages, although the magnitude of this benefit depended on life stage and on the specific pH and aluminum levels. Within the range of 0.5–8 mg Ca/L actual differences in survival across calcium concentrations were sometimes small. But in some

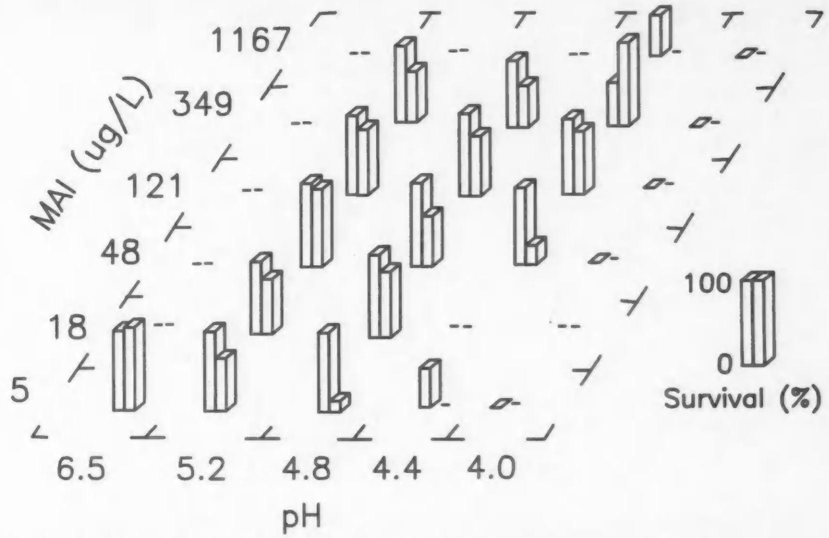

FIG. 8. Swim-up fry survival after a 21-d exposure to the designated combinations of pH and aluminum at 0.75 mg Ca/L (left bar) and 20-d recovery at pH 6.5 and 1.48 mg Ca/L (right bar). A dash indicates combinations of pH and aluminum that were not tested.

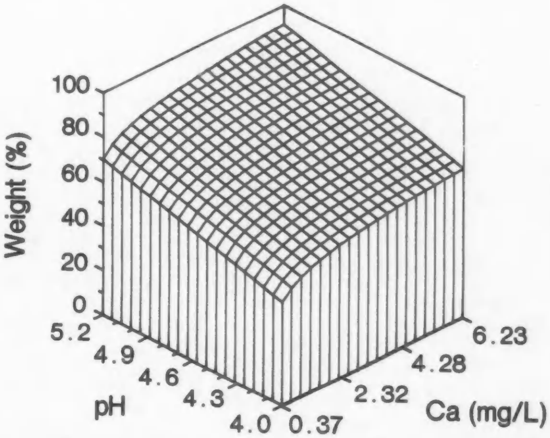

FIG. 9. Swim-up fry weight in the 21-d experiment predicted by the fitted regression model: Weight (mg) = 12.87 pH + 10.91 log(Ca) − 15.6 (R^2 = 0.63). All data were normalized to 66.2 mg (the maximum weight observed at pH 6.5, 6.23 mg Ca/L, 5 μg MAl/L).

cases, increasing calcium concentration was the difference between low and no survival. Weight of surviving fish was largely a function of calcium and pH exposure. Weight data from each of the exposures were quite similar, with minima at low calcium and low pH and maxima at high calcium and high pH. Aluminum concentration generally had a lesser effect on weight than on survival. Calcium has been demonstrated to be important to the survival or growth of fish stressed by either acid or aluminum (e.g. Hunn 1985). Perhaps most important, extremely low calcium concentrations (0.5 mg/L) can stress fish experiencing little stress from pH or aluminum alone. The most marked effects of low calcium concentration were found in the 0.5–1mg/L range. Similar results have been reported for brown trout, *Salmo trutta* (Brown 1982, 1983), rainbow smelt, *Osmerus mordax*, (Kostecki et al. 1985), and amphibians (Freda and Dunson 1985).

A reduction in mean weight might result from either lower growth rate or size-dependent mortality of larger individuals. We qualitatively observed rapid mortality of smaller embryos at low pH in the 91-d experiment, suggesting that a change in growth rate occurred and that this change was sufficient to overcome size-dependent mortality of smaller fish. At higher pH, fry also absorbed their yolk more quickly, were more active, swam up earlier, and consumed more food. Retarded yolk absorption (e.g. Menendez 1976), decreased activity (e.g. Cleveland et al. 1986), increased time to swim-up (e.g. Swarts et al. 1978) and reduced feeding (e.g. Hunn et al. 1987) are all commonly observed responses of fish to acid or aluminum exposure in the laboratory. However, field data on growth of early life stages in acidified systems are lacking (Baker 1984).

Hatching was often disrupted or delayed when eyed eggs were exposed to either low pH or elevated aluminum (see also Ingersoll et al. 1985; Ingersoll 1986). Fry would begin to break through the chorion, but died partially hatched, unable to escape. When these fish were taken from the egg cup, the chorion was easily removed, suggesting that these hatching problems resulted from decreased fry movement (e.g. Peterson et al. 1980). At the end of the 21-d eyed egg experiment, hatching was delayed and the chorion had not begun to break when the eggs were transferred to recovery water. However, most of these fish hatched within 48-h, suggesting hatching problems may also have resulted from decreased chorionase (a hatching enzyme) activity at low pH (e.g. Haya and Waiwood 1981; Waiwood and Haya 1983). Similar results have been reported for brook trout (e.g. Kwain and Rose 1985), rainbow trout (Nelson 1982), and Atlantic salmon, (e.g. Johansson et al. 1977; Skogheim and Rosseland 1984). Hatching effects were observed in the 21-d experiment started with eyed eggs, but not in the 91-d experiment started with freshly fertilized eggs. The lack of a delay in hatching with freshly fertilized eggs could have resulted from selective mortality of individual eggs that would eventually hatch if they had survived exposure to low pH. A second explanation for the lack of delayed hatching is that the chorion may have been weakened by exposure to chronic acid conditions, making hatching easier for fry incubated in low pH water.

To understand how acidified natural waters influence fish populations, results from laboratory studies (e.g. survival, growth, and reproduction) must be related to the response of fish populations. Two models have been developed by Van Winkle et al. (1986) that link data from our laboratory exposures to the response of fish populations in acid-sensitive waters. The first model (PHALCA) estimates the number of fish surviving as a function of time at given pH, aluminum, and calcium exposure levels. The second model (FISHEGGS) evaluates the reduction in reproductive potential (e.g. Mount et al. 1988) in fish populations attributable to the effects of pH, aluminum, and calcium on survival of yearling fish, survival of older fish, and fecundity. When predictions are generated using these models (Christensen et al. 1988), two essential factors need to be considered: (1) the specific life stage that may be exposed and (2) the exposure duration in the field. For example, freshly fertilized and eyed eggs were quite sensitive to pH, aluminum, or calcium exposure. Yet yolk-sac and swim-up fry may be the only early life stages of brook trout to be exposed to elevated acidity and aluminum in the field. Increased acidity and aluminum are often associated with spring snow melt or fall rain storms (Leivestad et al. 1976) and short-term pulse exposure to these acidic conditions may often be more toxic to fish than continuous exposure (Siddens et al. 1986; Gunn and Noakes 1987; Mount et al. 1990). These episodic events often coincide with spawning or embryogenesis (freshly fertilized and eyed eggs) or with emergence of young fry (yolk-sac and swim-up fry) (Peterson et al. 1982; Gunn 1986). Fall-spawning brook trout often select redds with more alkaline groundwater upwelling, which may ensure a satisfactory environment for development through egg and larval stages (e.g. Webster and Eiriksdottir 1976; Gunn and Keller 1984). However, at swim-up, fry may be subjected to potentially toxic elevations in acidity and aluminum between the surface water and the interstitial water of the spawning redd (Schofield 1976; Gunn 1986; Gunn and Noakes 1986). Clearly, the timing, duration, frequency, and magnitude of field exposure must be considered to fully assess the susceptibility of brook trout populations to acidification.

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References

Baker, J. P. 1984. Fishes, p. 5–74 to 5–139. *In* A. A. Altshuller and R. A. Linthurst [ed.] The acid deposition phenomenon and its effects; Critical Assessment Review Papers. Vol II. Effects Sciences. EPA-600/8-83-016B. U.S. Environmental Protection Agency. Washington, DC.

Baker, J. P., and C. L. Schofield. 1982. Aluminum toxicity to fish in acidic water. Water Air Soil. Pollut. 18: 289–309.

Beamish, R. J., and H. H. Harvey. 1972. Acidification of the La Cloche Mountain Lakes, Ontario, and resulting fish mortalities. J. Fish. Res. Board Can. 29: 1131–1143.

Beamish, R. J., W. L. Lockhart, J. C. Van Loon, and H. H. Harvey. 1975. Long-term acidification of a lake and resulting effects on fishes. Ambio 4: 98–102.

Bergman, H. L., J. S. Mattice, and D. J. A. Brown. 1988. Lake acidification and fisheries project: adult brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1561–1562.

Bergman, H. L., and J. S. Mattice. 1990. Lake acidification and fisheries project; early life stage and juvenile brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 1578–1579.

Brown, D. J. A. 1981. The effects of various cations on the survival of brown trout, *Salmo trutta* at low pHs. J. Fish. Biol. 18: 31–40.

1982. Influence of calcium on the survival of eggs and fry of brown trout (*Salmo trutta*) at pH 4.5. Bull. Environ. Contam. Toxicol. 28: 664–668.

1983. Effect of calcium and aluminum concentrations on the survival of brown trout (*Salmo trutta*) at low pH. Bull. Environ. Contam. Toxicol. 30: 582–587.

Christensen, S. W., J. E. Breck, and W. Van Winkle. 1988. Predicting acidification effects on fish populations, using laboratory data and field information. Environ. Toxicol. Chem. 7: 735–747.

Cleveland, L., E. E. Little, S. J. Hamilton, D. R. Buckler, and J. B. Hunn. 1986. Interactive toxicity of aluminum and acidity to early life stages of brook trout. Trans. Am. Fish. Soc. 115: 610–620.

Craig, G. R., and W. F. Baksi. 1977. The effects of depressed pH on flagfish reproduction, growth and survival. Water Res. 11: 621–626.

Cronan, C. S., and C. L. Schofield. 1979. Aluminum leaching response to acid precipitation: Effects on high-elevation watersheds in the northeast. Science (Wash., DC) 204: 304–306.

Dalziel, T. R. K., R. Morris, and D. J. A. Brown. 1986. The effects of low pH, low calcium concentrations and elevated aluminum concentrations on sodium fluxes in brown trout, *Salmo trutta* L. Water Air Soil Pollut. 30: 569–577.

Daye, P. G. 1980. Attempts to acclimate embryos and alevins of Atlantic salmon, *Salmo salar*, and rainbow trout, *S. gairdneri*, to low pH. Can. J. Fish. Aquat. Sci. 37: 1035–1038.

Daye, P. G., and E. T. Garside. 1977. Lower lethal levels of pH for embryos and alevins of Atlantic salmon, *Salmo salar* L. Can. J. Zool. 55: 1504–1508.

1979. Development and survival of embryos and alevins of the Atlantic salmon, *Salmo salar* L., continuously exposed to acidic levels of pH, from fertilization. Can. J. Zool. 57: 1713–1718.

Dixon, W. J., M. B. Brown, L. Engelman, J. W. Frane, M. A. Hill, R. I. Jennrich, and J. D. Toporek. 1985. BMDP statistical software. Univ. California Press, Berkeley, CA. 734 p.

Driscoll, C. T. 1980. Aqueous speciation in the Adirondack region of New York State, USA, p. 214–215. *In* D. Drablos and A. Tollan [ed.] Ecological impact of acid precipitation: proceedings of an international conference Sandefjord, Norway, March 11–14, 1980. SNSF-project, As-NLH, Norway.

Driscoll, C. T., J. P. Baker, J. J. Bisogni, and C. L. Schofield. 1980. Effect of aluminum speciation on fish in dilute acidified waters. Nature (Lond.) 284: 161–164.

Fivelstad, S., and H. Leivestad. 1984. Aluminum toxicity to Atlantic salmon (*Salmo salar* L.) and brown trout (*Salmo trutta* L.): mortality and physiological response, p. 69–77. Institute of Freshwater Research, National Swedish Board of Fisheries. Rep. 61.

Freda, J., and W. A. Dunson. 1985. The influence of external cation concentration on the hatching of amphibian embryos in water of low pH. Can. J. Zool. 63: 2649–2656.

Frennette, J., and J. J. Dodson. 1984. Brook trout (*Salvelinus fontinalis*) population structure in acidified Lac Tantare, Quebec. Can. J. Fish. Aquat. Sci. 41: 865–877.

Gunn, J. M. 1986. Behaviour and ecology of salmonid fishes exposed to episodic pH depressions. Environ. Biol. Fish. 17: 241–252.

Gunn, J. M., and W. Keller. 1984. Spawning site water chemistry and lake trout (*Salvelinus namaycush*) sac fry survival during spring snowmelt. Can. J. Fish. Aquat. Sci. 41: 319–329.

Gunn, J. M., and D. L. G. Noakes. 1986. Avoidance of low pH and elevated Al concentrations by brook charr (*Salvelinus fontinalis*) alevins in laboratory tests. Water Air Soil Pollut. 30: 497–503.

1987. Latent effects of pulse exposure to aluminum and low pH on size, ionic composition, and feeding efficiency of Lake Trout (*Salvelinus namaycush*) alevins. Can. J. Fish. Aquat. Sci. 44: 1418–1424.

Harvey, H. H. 1979. The acid deposition problem and emerging research needs in the toxicology of fishes, p. 115–128. Proc. Fifth Annual Aquatic toxicity Workshop, Hamilton, Ontario, Nov. 7–9, 1978.

Haya, K., and B. A. Waiwood. 1981. Acidic pH and chorionase activity of Atlantic salmon (*Salmo salar*). Bull. Environ. Contam. Toxicol. 27:7–12.

Hunn, J. B. 1985. Role of calcium in gill function in freshwater fishes. Comp. Biochem. Physiol. 82A: 543–547.

Hunn, J. B., L. Cleveland, and E. E. Little. 1987. Influence of pH and aluminum on developing brook trout in a low calcium water. Environ. Pollut. 43: 63–73.

Hutchinson, N. J., and K. E. Holtze. 1985. Biological effects of acidification. XI. Factors influencing the acute lethality of hydrogen and Al ion to lake and brook trout, p. 104. The International Symposium on Acid Precipitation. Muskoka conference '85. Muskoka, Ontario, 15–20 September 1985, 428 p.

Ingersoll, C. G. 1986. The effects of pH, aluminum, and calcium on survival and growth of brook trout (*Salvelinus fontinalis*) early life stages. PhD. thesis, The University of Wyoming, Laramie, WY. 122 p.

Ingersoll, C. G., T. W. La Point, H. L. Bergman, and J. Breck. 1985. An early life stage brook trout (*Salvelinus fontinalis*) experiment to determine the effects of pH, calcium, and aluminum in low conductivity water, p. 42–48. *In* Rago, P. J., and R. K. Schreiber [ed.] Symp. Proc., 114th Ann. Meet., Am. Fish. Soc., Biological Rep. 80 (40.21): 106 p.

Ingersoll, C. G., D. D. Gulley, D. R. Mount, M. E. Mueller, J. R. Hockett, J. Fernandez, and H. L. Bergman. 1990b. Aluminum and acid toxicity to two strains of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 1641–1648.

Ingersoll, C. G., D. A. Sanchez, J. S. Meyer, D. D. Gulley, and J. E. Tietge. 1990a. Epidermal response to pH, aluminum, and calcium exposure in brook trout (*Salvelinus fontinalis*) fry. Can. J. Fish. Aquat. Sci. 47: 1616–1622.

Jagoe, C. H., T. A. Haines, and F. W. Kircheis. 1984. Effects of reduced pH on three life stages of Sunapee char *Salvelinus alpinus* Bull. Environ. Contam. Toxicol. 33: 430–438.

Jensen, K. W., and E. Snekvik. 1972. Low pH levels wipe out salmon and trout populations in southernmost Norway. Ambio 1: 223–225.

Johansson, N., P. Runn, and G. Milbrink. 1977. Early development of three salmonid species in acidified water. Zoon 5: 127–132.

Kostecki, P. T., K. R. Byrne, and E. J. Calabrese. 1985. Reproductive failure due to environmental pH and ionic factors in landlocked rainbow smelt (*Osmerus mordax*). Water Resour. Res. Cent. Univ. of Mass., Amherst, MA. Pub. 150: 30 p.

Kwain, W., and G. A. Rose. 1985. Growth of brook trout *Salvelinus fontinalis* subject to sudden reductions of pH during their early life history. Trans. Am. Fish. Soc. 114: 564–570.

LaZerte, B. 1984. Forms of aqueous aluminum in acidified catchments of southern Ontario: a methodological analysis. Can. J. Fish. Aquat. Sci. 41: 766–776.

Lee, R. M., and S. D. Gerking. 1980. Survival and reproductive performance of the desert pupfish, *Cyprinodon n. nevadensis* (Eigenmann and Eigenmann), in acid waters. J. Fish. Biol. 17: 507–515.

Leivestad, H., G. Hendrey, I. P. Muniz, and E. Snekvik. 1976. Effects of acid precipitation on freshwater organisms, p. 87–111. *In* F. H. Braekke [ed.] Impact of acid precipitation of forest and freshwater ecosystems in Norway. SNSF Project, Report FR-6/76.

Lockhart, W. L., and A. Lutz. 1977. Preliminary biochemical observations of fishes inhabiting an acidified lake in Ontario, Canada. Water Air Soil Pollut. 7: 317–332.

Marcus, M. D., B. R. Parkhurst, J. P. Baker, C. S. Creager, T. E. Fannin, C. G. Ingersoll, D. R. Mount, and F. J. Rahel. 1986. A critical evaluation and compilation of reported effects of acidification on aquatic biota. Vol. I: Compiled data and Vol. II: Evaluation forms. Res. Rep., EPRI EA-2346. Electric Power Research Institute, Palo Alto, CA.

Menendez, R. 1976. Chronic effects of reduced pH on brook trout (*Salvelinus fontinalis*). J. Fish. Res. Board Can. 33: 118–123.

Mount, D. I. 1973. Chronic effect of low pH on fathead minnow survival, growth and reproduction. Water Res. 7: 987–993.

Mount, D. I., and W. A. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. Water Res. 1: 21–29.

Mount, D. R., C. G. Ingersoll, D. D. Gulley, J. D. Fernandez, T. W. La Point, and H. L. Bergman. 1988. The effects of long-term exposure to acid, aluminum, and low calcium on adult brook trout (*Salvelinus fontinalis*): 1. Survival, growth, fecundity, and progeny survival. Can. J. Fish. Aquat. Sci. 45: 1623–1632.

Mount, D. R., M. J. Swanson, J. E. Breck, A. M. Farag, and H. L. Bergman. 1990. Responses of brook trout (*Salvelinus fontinalis*) fry to fluctuating acid, aluminum, and low calcium exposure. Can. J. Fish. Aquat. Sci. 47: 1623–1630.

Muniz, I. P., and H. Leivestad. 1980a. Toxic effects of aluminum on the brown trout, *Salmo trutta*, p. 320–321. *In* D. Drablos and A. Tollan [ed.] Ecological impact of acid precipitation: proceedings of an international conference Sandefjord, Norway, March 11–14, 1980. SNSF-project,As-NLH, Norway.

1980b. Acidification-effects on freshwater fish, p. 84–92. *In* D. Drablos and A. Tollan [ed.] Ecological impact of acid precipitation: proceedings of an international conference Sandefjord, Norway, March 11–14, 1980. SNSF-project, As-NLH, Norway.

Nelson, J. A. 1982. Physiological observations on developing rainbow trout, *Salmo gairdneri* (Richardson), exposed to low pH and varied calcium ion concentrations. J. Fish. Biol. 20: 359–372.

Neter, J., W. Wasserman, and M. H. Kutner. 1985. Applied linear statistical models. Richard D. Irwin, Inc., Homewood, IL. 1127 p.

Nie, N. H., C. H. Hull, J. G. Jenkins, K. Steinbrenner, and D. H. Bent. 1975. SPSS Statistical package for the social sciences. McGraw–Hill, New York, NY. 675 p.

Parkhurst, B. R. 1987. A comparison of laboratory and in situ bioassays for evaluating the toxicity of acidic water to brook trout. PhD. thesis, University of Wyoming, Laramie, WY. 101 p.

Parkhurst, B. R., H. L. Bergman, J. D. Fernandez, D. D. Gulley, J. R. Hockett, and D. A. Sanchez. 1990. A comparison of laboratory and in situ bioassays for evaluating the toxicity of acidic waters to brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. (In press)

Peterson, R. H., P. G. Daye, and J. L. Metcalfe. 1980. The effects of low pH on hatching of Atlantic salmon eggs, p. 238. *In* D. Drablos and A. Tollan [ed.] Ecological impact of acid precipitation: proceedings of an international conference Sandefjord, Norway, March 11–14, 1980. SNSF-project, As-NLH, Norway.

Peterson, R. H., P. G. Daye, G. L. Lacroix, and E. T. Garside. 1982. Reproduction in fish experiencing acid and metal stress, p. 177–196. *In* R. Johnson [ed.] Acid Rain/Fisheries. Northeastern Division American Fisheries Society, Bethesda, MD.

Rask, M. 1984. The effect of low pH on perch, *Perca fluviatilis* L. II. The effect of acid stress on different development stages of perch. Ann. Zool. Fenn. 21: 9–13.

Rombough, P. J. 1982. Effects of low pH on eyed embryos and alevins of Pacific salmon. Can. J. Fish. Aquat. Sci 40: 1575–1582.

Rosseland, B. O., I. Sevaldrud, D. Svalastog, and I. P. Muniz. 1980. Studies on freshwater fish populations — effects of acidification on reproduction, population structure, growth and food selection, p. 336–337. *In* D. Drablos and A. Tollan [ed.] Ecological impact of acid precipitation: proceedings of an international conference Sandefjord, Norway, March 11–14, 1980. SNSF-project, As-NLH, Norway.

Rosseland, B. O., and O. K. Skogheim. 1984. A comparative study on salmonid fish species in acid aluminum-rich water. II. Physiological stress and mortality of one- and two-year-old fish, p. 186–194. Inst. Freshwater Res. National Swedish Board of Fisheries. Rep. 61.

Schofield, C. L. 1976. Acid precipitation: effects on fish. Ambio 5: 228–230.

Schofield, C. L. 1982. Historical fisheries changes in the Unied States related to surface water pH, p. 57–67. *In* R. Johnson [ed.] Acid Rain/Fisheries. Northeastern Division American Fisheries Society. Bethesda, MD.

Schofield, C. L., and J. R. Trojnar. 1980. Aluminum toxicity to brook trout (*Salvelinus fontinalis*) in acidified waters, p. 341–366. *In* T. Y. Toribara, M. W. Miller, and P. E. Morrow [ed.] Polluted Rain. Plenum Press, New York, NY.

Seip, H. M. 1980. Acidification of freshwater-sources and mechanisms, p. 358–366. *In* D. Drablos and A. Tollan [ed.]. Ecological impact of acid precipitation: proceedings of an international conference Sandefjord, Norway, March 11–14, 1980. SNSF-project, As-NLH, Norway.

Sevaldrud, I. H., and O. K. Skogheim. 1986. Changes in fish populations in southernmost Norway during the last decade. Water Air Soil Pollut. 30: 381–386.

Siddens, L., W. K. Seim, L. R. Curtis, and G. A. Chapman. 1986. Comparison of continuous and episodic exposure to acidic, aluminum-contaminated waters of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 43: 2036–2040.

Skogheim, O. K., and B. O. Rosseland. 1984. A comparative study on salmonid fish species in acid aluminum-rich water. I. Mortality of eggs and alevins, p. 177–185. Inst. Freshwater Res. National Swedish Board of Fisheries. Rep. 61.

Sokal, R. R., and F. J. Rohlf. 1981. Biometry. W. H. Freeman and Co., San Francisco, CA. 859 p.

Swarts, F. A., W. A. Dunson, and J. E. Wright. 1978. Genetic and environmental factors involved in increased resistance of brook trout to sulfuric acid solutions and mine acid polluted waters. Trans. Am. Fish. Soc. 107: 651–677.

TROJNAR, J. R. 1977a. Egg hatchability and tolerance of brook trout (*Salvelinus fontinalis*) fry at low pH. J. Fish. Res. Board Can. 34: 574–579.

1977b. Egg and larval survival of white suckers (*Catostomus commersoni*) at low pH. J. Fish. Res. Board Can. 34: 262–266.

VAN WINKLE, W., S. W. CHRISTENSEN, AND J. E. BRECK. 1986. Linking laboratory and field responses of fish populations to acidification. Water Air Soil Pollut. 30: 639–648.

WAIWOOD, B. A., AND K. HAYA. 1983. Levels of chorionase activity during embryonic development of *Salmo salar* under acid conditions. Bull. Environ. Contam. Toxicol. 30: 511–515.

WEBSTER, D. A., AND G. EIRIKSDOTTIR. 1976. Upwelling water as a factor influencing the choice of spawning sites by brook trout (*Salvelinus fontinalis*). Trans. Am. Fish. Soc. 104: 416–421.

WOOD, C. M., D. G. MCDONALD, C. G. INGERSOLL, D. R. MOUNT, O. E. JOHANNSSON, S. LANDSBURGER, AND H. L. BERGMAN. 1990a. Effects of water acidity, calcium, and aluminum on whole body ions of brook trout continuously exposed from fertilization to swim-up: a study by instrumental neutron activation analysis. Can. J. Fish. Aquat. Sci. 47: 1593–1603.

1990b. Whole body ions of brook trout alevins: responses of yolk-sac and swim-up stages to water acidity, calcium, and aluminum, and recovery effects. Can. J. Fish. Aquat. Sci. 47: 1604–1615.

WRIGHT, R. F., AND E. T. GJESSING. 1976. Changes in the chemical composition of lakes. Ambio 5: 219–223.

WRIGHT, R., AND E. SNEKVIK. 1978. Acid precipitation: chemistry and fish populations in 700 lakes in southern most Norway. Verh. Int. Vere. Limnol. 20: 765–775.

Effects of Water Acidity, Calcium, and Aluminum on Whole Body Ions of Brook Trout (*Salvelinus fontinalis*) Continuously Exposed from Fertilization to Swim-Up: A Study by Instrumental Neutron Activation Analysis

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Water Ca, rather than pH or Al, was the most important factor affecting whole body electrolyte levels in fry exposed from fertilization to swim-up (91 d) to 84 combinations of pH (6.5, 5.2, 4.8, 4.4, 4.0), Ca (0.5, 1, 2, 8 mg/L), and Al (0, 12, 37, 111, 333, 1000 μg/L) in flowing soft water. Aluminum accumulation occurred only at water Al levels > 111 μg/L; Al accumulation was inhibited both by increasing Ca and decreasing pH. Under control conditions (pH = 6.5, Ca = 2 mg/L, Al = 0 μg/L), whole body Na, Cl, K, and Ca levels all increased greatly during development, while Mg decreased. Body Ca levels were elevated up to 3-fold, and Na, Cl, and K up to 2-fold by increasing water Ca at the same pH and Al. Low pH had a small negative influence, intermediate levels of Al (37, 111) a slight positive influence, and higher levels of Al a negative influence on Na, Cl, K, and Ca levels. Whole body Mg showed opposite trends, reflecting delayed development under adverse conditions. At pH = 6.5, the positive influence of increasing water Ca on most whole body ions showed a clear threshold between 0.5 and 1 mg/L. At lower pH, this threshold was shifted to between 2 and 8 mg/L, indicating that Ca levels sufficient to support healthy development at circumneutral pH may prove inadequate under acidified conditions.

Le calcium dans l'eau, plutôt que le pH ou l'Al, constitue le plus important facteur à agir sur l'équilibre des électrolytes chez l'alevin exposé depuis la fécondation jusqu'à la nage (91 jours) à 84 combinaisons de pH (6,5, 5,2, 4,8, 4,4, 4,0), de conc. de Ca (0,5, 1, 2, 8 mg/L) et d'Al (0, 12, 37, 111, 333, 1000 μg/L) dans une eau douce en circulation. L'accumulation d'Al s'est produite seulement à des concentrations d'Al dans l'eau sup. à 111 μg/L; l'accumulation d'Al était inhibée à la fois par l'augmentation de la conc. en Ca et l'abaissement du pH. Dans les conditions de référence (pH = 6,5, Ca = 2 mg/L, Al = 0 μg/L;), la conc. dans l'organisme de Na, Cl, K et Ca a considérablement augmenté au cours du développement alors que celle du Mg s'est abaissée. La conc. de Ca a augmenté de trois fois alors que celles des Na, Cl et K ont augmenté de deux fois lorsque la teneur en Ca de l'eau a été accrue et que le pH et la conc. en Al ont été gardés constants. Les pH acides avaient un léger effet négatif, les conc. intermédiaires d'Al (37, 111) avaient un léger effet positif et les conc. élevées d'Al avaient un effet négatif sur la conc. de Na, Cl, K et Ca. La conc. en Mg dans l'organisme variait en sens inverse, ce qui traduisait un développement retardé par des conditions adverses. À pH = 6,5, on voyait apparaître nettement un seuil entre 0,5 et 1 mg/L dans l'influence positive de l'augmentation de conc. en Ca dans l'eau sur la plupart des électrolytes. À pH acide, ce seuil passait entre 2 et 8 mg/L, ce qui indique que des conc. en Ca suffisantes pour assurer un développement sain des organismes à pH situé dans la page de neutralité, peuvent se révéler insuffisantes en milieu acide.

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In juvenile and adult salmonids, ionoregulatory disturbance is the key toxic mechanism causing death in acute acid stress. This is further exacerbated by the presence of aluminum (Al) but ameliorated by calcium (Ca) (Leivestad 1982; Wood 1989; McDonald et al. 1989). Ionoregulatory disturbance may also occur during chronic sublethal exposures, but here the situation may be complicated by acclimation and/or recovery, and by protective rather than toxic effects of Al (Wood et al. 1988).

Early life stages are more sensitive to acid/aluminum exposure than juveniles and adults; mortality over the fertilization through swim-up period may be an important cause of recruitment failure (Haines 1981; Howells 1984; Gunn 1986). A number of studies have indicated that intense ionic uptake from the water must occur at this time to achieve the rapid mineralization of the body which accompanies early development (McCayet al. 1936; Hayes et al. 1946; Zeitoun et al. 1976; Shen and Leatherland, 1978a; Talbot et al. 1982; Rombough and Garside, 1984). It is therefore surprising that the effects of acid/Al stress on ionoregulation in these early life stages have received little experimental attention. One notable exception is the work of Peterson et al. (1982) and Peterson and Martin–Robichaud (1986) on larval Atlantic salmon (*Salmo salar*) chronically exposed to acid stress alone. Pronounced reductions in Na, Ca, and K accumulation during development were documented. The effects of water Al or Ca, of exposure at different developmental stages, and of recovery were not tested. However, very recently, Gunn and Noakes (1987) have examined some of these topics after recovery from pulse acid/Al exposures in alevins of lake trout (*Salvelinus namaycush*).

The toxicological experiments described in the preceding paper (Ingersoll et al. 1990) on early life stages of brook trout (*Salvelinus fontinalis*) offered a unique opportunity to evaluate these problems in an extensive pH/Ca/Al matrix. We have employed instrumental neutron activation analysis (INAA) to measure whole body levels of Na, Cl, K, Ca, Mg, and Al in surviving fry from these experiments. INAA, which has recently been employed in other acidification studies (Fraser and Harvey 1984; Cunningham and Shuter 1986; Gunn and Noakes 1987), was a particularly suitable method for these very small samples (~5 – 15 mg dry weight). The technique provides sensitive, simultaneous, and non-destructive multi-element analysis without extensive sample preparation (Heydorn 1984).

The present paper reports the ionoregulatory effects seen in surviving brook trout after a continuous 91 d exposure (from fertilization through swim-up) to an 84 cell matrix of pH, Ca, and Al. This matrix covers the total range of occurrence of these three parameters in acid-sensitive soft waters of eastern North America. The accompanying paper (Wood et al. 1990) documents the effects on previously unexposed yolk-sac fry and swim-up fry of shorter term challenges and recoveries in this same matrix.

Materials and Methods

Exposures

Details of the fish and the exposure regimes are given by Ingersoll et al. (1990). In brief, all fish came from a single batch of 26 000 freshly fertilized eggs (from 40 females and 40 males) spawned in soft water (pH $\simeq$6.5, Ca $\simeq$ 2 mg/L). The exposure matrix consisted of 84 combinations (nominal values) of pH (6.5, 5.2, 4.8, 4.4, 4.0), Ca (0.5, 1, 2, 8 mg/L), and Al (0, 12, 37, 111, 333, 1000 μg/L) in flowing artificial softwater (Na $\simeq$ 4, Cl $\simeq$ 8 mg/L) acidified with H_2SO_4. Temperature was 10.9°C, close to the reported optimum (12.4°C) for the growth and survival of brook trout alevins (McCormick et al. 1972), and photoperiod 12-h light/12-h darkness. Fry were offered Rangens Trout Starter (Rangens Inc., Buhl, Idaho), and exposure chambers were cleaned daily; no substrate was employed in the chambers. Exogenous feeding started between days 55 and 60 post-fertilization; it was not possible to estimate the amount of food actually eaten.

The water chemistry data from these exposures have been reported in detail (see table 1 of Ingersoll et al. 1990). In brief, pH and Ca levels were recorded daily and other parameters weekly or more frequently on water samples taken directly from the exposure chambers. Concentrations were generally very stable, with coefficients of variation typically <20%. On an absolute basis, measured Ca averaged about 25% less than nominal concentrations, while measured pH and Al (both total Al and monomeric Al) were in close agreement with nominal values. Nominal values have been used throughout for data analysis and tabulation.

The longest exposure, which is the focus of the present paper, started on day 0 with freshly fertilized eggs and continued for 91 d through swim-up. On day 91, every cell was sampled for INAA except where this was impossible due to mortality (see below). In addition, two 21 d age-specific challenge and 20 d recovery experiments were performed with yolk-sac fry (over days 49–90) and swim-up fry (over days 70–111; see Wood et al. 1990). In both, the fish had been raised at pH = 6.5, Ca = 2 mg/L, Al = 0 μg/L prior to challenge in the matrix, and were returned to this condition for recovery. These experiments provided some of the baseline data on temporal changes in body composition, summarized in Fig. 1.

Analyses

Typically, 8–10 survivors out of the original 50 fish in each cell were sacrificed for INAA, though this was reduced in cases where survival was low. The smallest number ever taken for INAA was 4. Cells with fewer survivors were not analysed. At sacrifice, fish were individually blotted dry, weighed (0.1 mg accuracy) and then dried to a constant weight at 65°C in an INAA vial. While both wet and dry weights were employed in data analysis (see Results), elemental concentrations have been routinely expressed in terms of wet weight. This is in accord with the recommendations of Shearer (1984) and the fact that wet weight measurements were intrinsically more accurate in these very small fish. For example, a 1% error in water content would be associated with a 5–10% error in dry weight.

Instrumental neutron activation analysis was carried out at the McMaster University Nuclear Reactor. Samples were irradiated with thermal neutrons (flux density = $5 \times 10^{12} n\mathrm{cm}^{-2} \cdot \mathrm{sec}^{-1}$), and after a 1 min delay, were counted for 10 min using a hyper-pure germanium detector coupled to a Canberra multichannel analyzer (Series 40 or 90). The peaks of interest and their energies (keV) were ^{24}Na (1368.4), ^{38}Cl (1642.2), ^{42}K (1524.6), ^{49}Ca (3084.4), ^{27}Mg (1014.5), and ^{28}Al (1778.9). Concentrations were calculated by comparison to known standards using routine equations (Desote et al. 1972).

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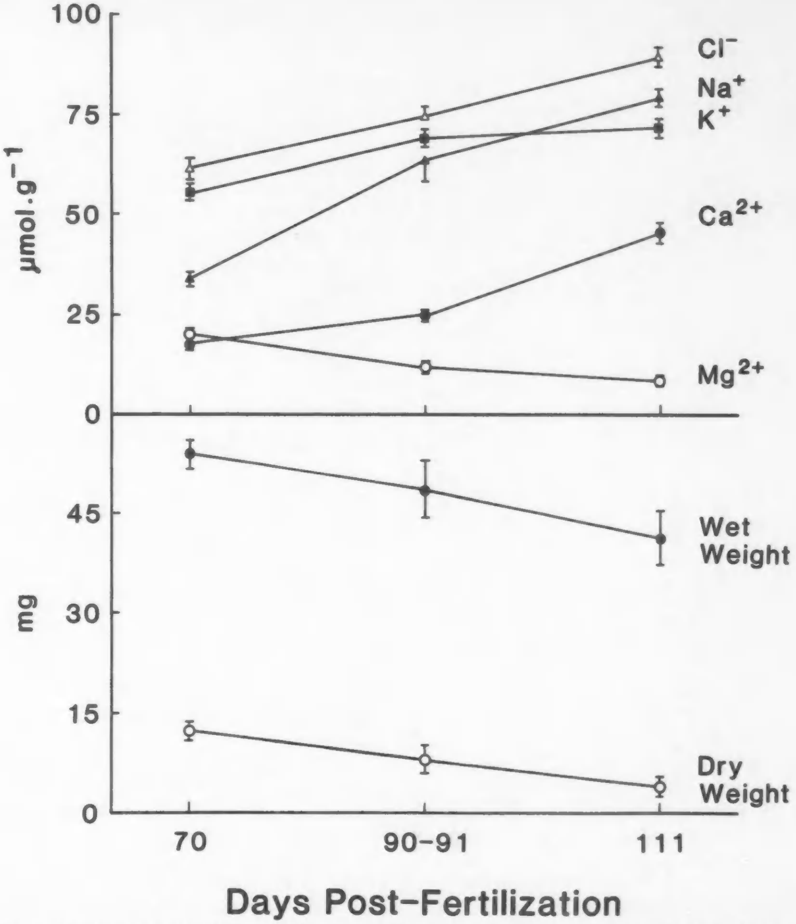

FIG. 1. Temporal changes in wet weight, dry weight, and the whole body concentrations (wet weight basis) of Cl, Na, K, Ca, and Mg in brook trout fry during early development under control conditions (pH=6.5, Ca=2 mg/L, Al=0 µg/L). Means ± 1SEM for *n*=20 at day 70, *n*=27 at days 90–91, and *n*=9 at day 111.

The NBS citrus leaf standard #1572 was used as a primary standard; the NRC marine fish tissue reference standard gave essentially identical results. As a check on the reproducibility of the analyses, a homogeneous pool of dried trout tissue was prepared, and 6–9 separate samples analysed. The coefficients of variation were: Na=3.6%; Cl=9.7%; K=7.8%; Ca=11.9%; and Mg=9.8%. As a check on absolute accuracy, the samples were spiked with known amounts of atomic absorption standards (BDH, Harleco) sufficient to approximately double the native concentrations. Standard recoveries ±1 SD (by subtraction) were: Na=100.7 ± 10.0%; Cl=103.7 ± 3.0%; K=92.0 ± 6.5%; Ca=92.6 ± 9.4%; and Mg=113.0 ± 11.4%.

Aluminum analyses by INAA are complicated by the conversion of ^{31}P to ^{28}Al during irradiation. While there is approximately 2000 times more P than Al in uncontaminated fish (Shearer 1984), INAA is fortunately over 1000 times more sensitive to Al than to P because of the correspondingly higher conversion efficiency. We therefore adopted an empirical approach, calibrating the Al/P radioactivity of experimental samples against known Al standards. The mean "Al/P concentrations" of all fish in a particular treatment from Al=0 cells (i.e. no Al exposure) were subtracted from the Al-exposed fish to give mean "ΔAl concentrations." The assumption here was that the P concentration was relatively constant across treatments. This assumption appears valid as variation in "Al/P concentrations" between groups over wide ranges of water pH and Ca (at Al=0) was generally less than 10%. Based on these considerations, the detection limit for Al accumulation was a ΔAl of approximately 0.3 µmol/g, which corresponded to a 20–30% elevation in radioactivity for "Al/P concentration" above that present in fish from the relevant Al=0 cells. The reproducibility for Δ Al was better than ±10% above 2 µmol/g, based on repeated irradiations.

Statistical Analyses

Within each cell, data were expressed as means ±1 SEM for *n*=8 – 10 fish generally, though for a few cells (<10%) the *n* was as low as 4. Over the complete matrix, the relative

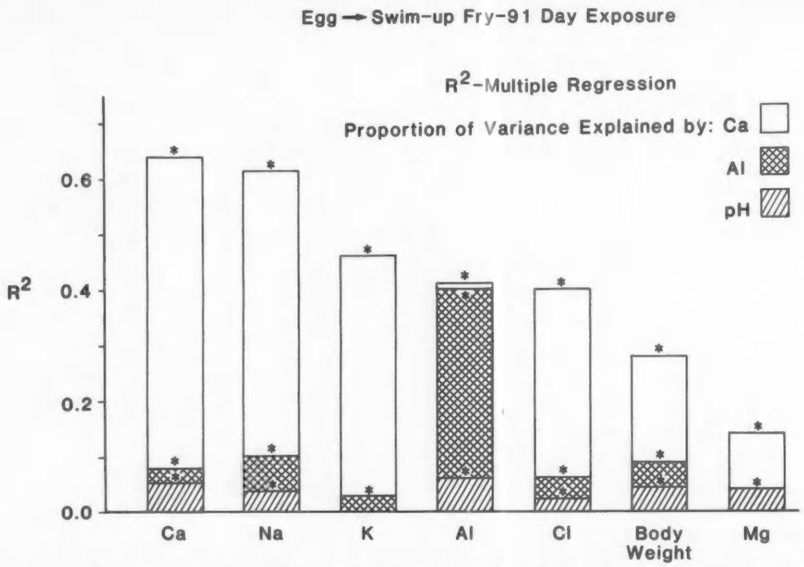

FIG. 2. Total variance explained (R^2) and proportion attributable to each factor (Ca, Al, pH), as determined by stepwise multiple regression analysis, for various whole body parameters in brook trout fry after 91 d continuous exposure to the matrix of pH, Ca, and Al. Asterisks indicate relationships which were also significant by factorial analysis of variance.

effects of pH, and Ca on whole body ion status and weight were first assessed using a factorial analysis of variance. The relative effects of the significant factors were then modelled, using stepwise multiple linear regression over the entire matrix. While relationships with pH appeared linear, marginal plots of the data suggested that some relationships with Ca and Al were not necessarily linear, and might be logarithmic. In addition, relationships with Al often appeared parabolic ($y = ax^2 + bx + c$), the greatest effects being seen at intermediate Al levels, with lesser effects at lower and higher levels. Therefore in these analyses both linear and logarithmic relationships were evaluated, and Al^2 and $(\log Al)^2$ terms were included in the regression program to permit Al to assume parabolic relationships. Factors were included in a regression only if their value of F to enter was significant ($p < 0.05$). In some instances, significant factors as determined by analysis of variance did not enter the regression equations. This would occur when their interaction effect(s) had entered with a more dominant factor. The opposite, a non-significant factor by analysis of variance entering the regression equation, occurred less often than predictable by chance alone. The final relationship accepted for each ion was the one which accounted for the maximum amount of variance. The total explained variance (R^2) and the proportion of that variance contributed by each factor (factor R^2) are summarized for each whole body parameter in Fig. 2. Asterisks indicate that the factor was also significant by factorial analysis of variance. Statistical analysis of the survival data has been reported by Ingersoll et al. (1990).

Results

Temporal Changes

As a background to this and the following study (Wood et al. 1990), Fig. 1 summarizes time-dependent changes in body composition of trout raised continuously under control conditions (pH = 6.5, Ca = 2mg/L, Al = 0 μg/L). This summary combines data sets taken at day 91 post-fertilization in the present study with those taken at days 70, 90 – 91, and 111 in several experiments described by Wood et al. (1990). All fish were raised simultaneously, and at any one time there were no significant differences among the different experiments. Exogenous feeding started between days 55 and 60; by day 70 the yolk-sac appeared to be about half resorbed, and by day 111 only a small remnant persisted.

From days 70 through 111, there was a small decline (~20%) in wet weight and a large decline (~60%) in dry weight as the alevins converted yolk into somatic tissue (Fig. 1). Thus water content increased considerably. During this same period, the concentrations of all measured whole body ions except Mg increased significantly (Fig. 1). The relative increases ranged from ~1.3-fold for K to ~3-fold for Ca. Only in the case of K could the reduction of wet body weight possibly explain the increase in concentration. Thus for Na, Cl, and Ca, there were real increases on a per individual basis as well as a body weight basis. In contrast, the concentration of Mg fell by ~60% between days 70 and 111.

Relative Influence of pH, Ca, and Al after 91 D

Fig. 2 summarizes the results of the multiple regression analyses for the major measured parameters. In every case, a factor significant by this analysis was also significant by factorial analysis of variance. The proportion of overall variance explained by the three measured variables Ca, Al, and pH ranged from over 60% for body Ca and Na concentrations down to about 14% for Mg concentration. For all parameters except ΔAl, water Ca was clearly the dominant influence after 91 d exposure, with water Al and pH playing relatively minor roles.

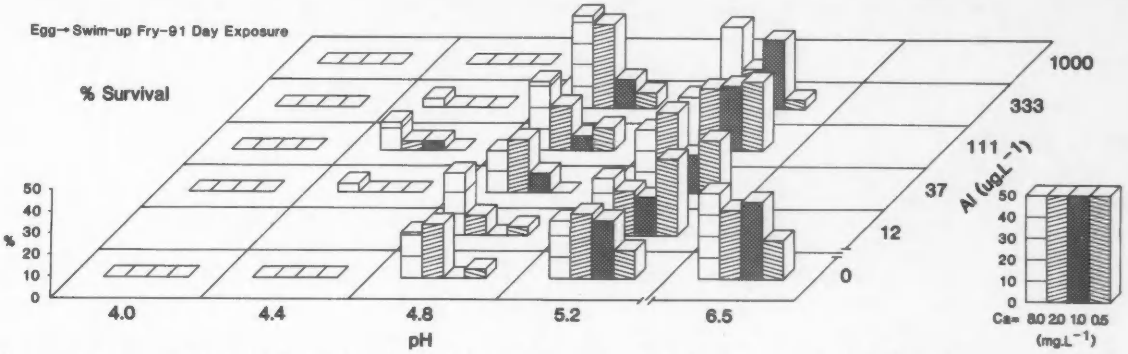

FIG. 3. Percentage survival of brook trout fry after 91 d continuous exposure to the designated matrix of pH, Ca, and Al from fertilization through swim-up. Each cell originally contained 50 eggs.

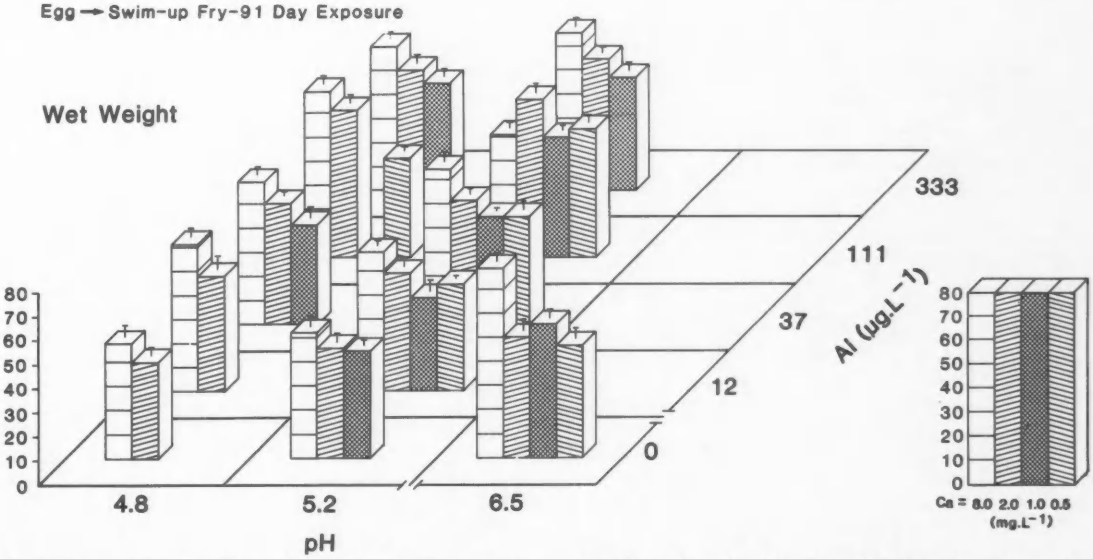

FIG. 4. Wet body weight of brook trout fry after 91 d continuous exposure to the designated matrix of pH, Ca, and Al. In cells not shown, data were not available due to low survival (cf. Fig. 3). Means ± 1 SEM for $n = 8-10$ fish in most cells, falling as low as four in cells with high mortality.

Mortality and Body Weights after 91 D

The relatively low survival (<50%; Fig. 3) even under control conditions was probably due to incomplete fertilization (see Ingersoll et al. 1990). At pH = 6.5 without Al, survival was strongly dependent on water Ca (Fig. 3). A similar protective effect of Ca was seen throughout the matrix, especially at pH = 4.8. Survival was negatively correlated with acidity; mortality was virtually complete at pH = 4.0 and 4.4. Al had no significant effect, though intermediate levels (Al = 37 − 111 µg/L) may have slightly ameliorated acid toxicity.

Wet body weight was also positively correlated with Ca (strongest with log Ca) and negatively correlated with acidity throughout the matrix (Fig. 2, 4). Al had a small influence in raising wet weight under acidic conditions. Dry body weight (not shown) was unaffected by Ca or Al, but was influenced in a biphasic fashion by pH, being greater at pH = 4.8 and 6.5 than at 5.2.

Whole Body Electrolytes after 91 D

Because of heavy mortality, fish were not available for analysis from some of the lower Ca cells at pH = 4.8 and 5.2, and from all cells at pH = 4.0 and 4.4. As some mortality occurred in every cell, the data are representative of the most resistant individuals in each group — i.e. after the process of selection has occurred.

In fish which had survived 91 d in the matrix, water Ca was clearly the dominant influence on the concentrations of all five whole body ions (Fig. 2), an effect which was pronounced even at pH = 6.5. Increasing Ca raised whole body levels of Na (Fig. 5) and Cl (Fig. 6), the two major extracellular electrolytes, in a concentration-dependent fashion in virtually every cell of the matrix (Fig. 2). The effect of Ca = 8 mg/L was particularly pronounced, increasing Na and Cl levels up to 2-fold above those at Ca = 0.5 mg/L. In addition, low pH had a very

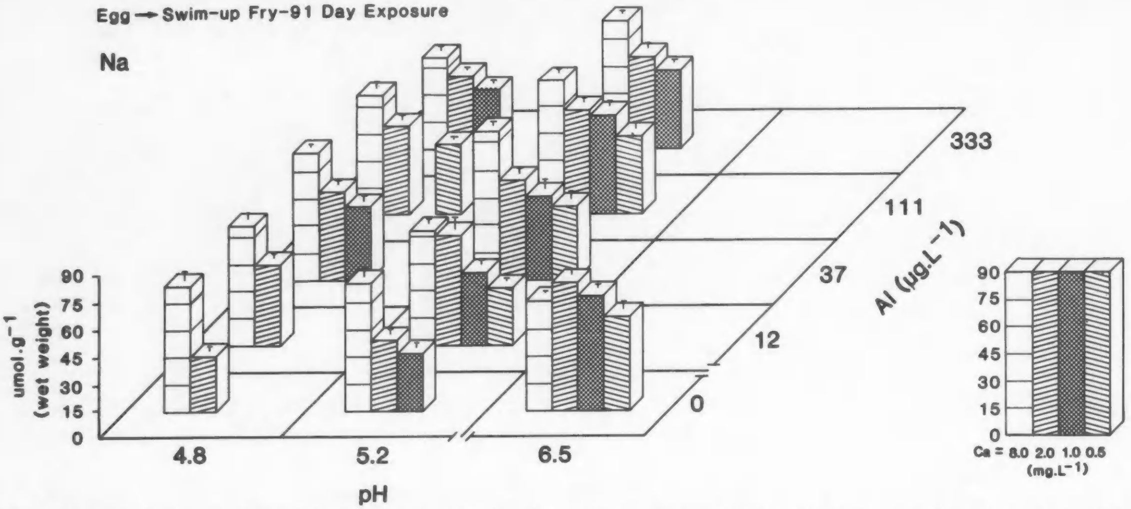

FIG. 5. Whole body concentrations of Na in brook trout fry after 91 d continuous exposure to the designated matrix of pH, Ca, and Al. Other details as in legend of Fig. 4.

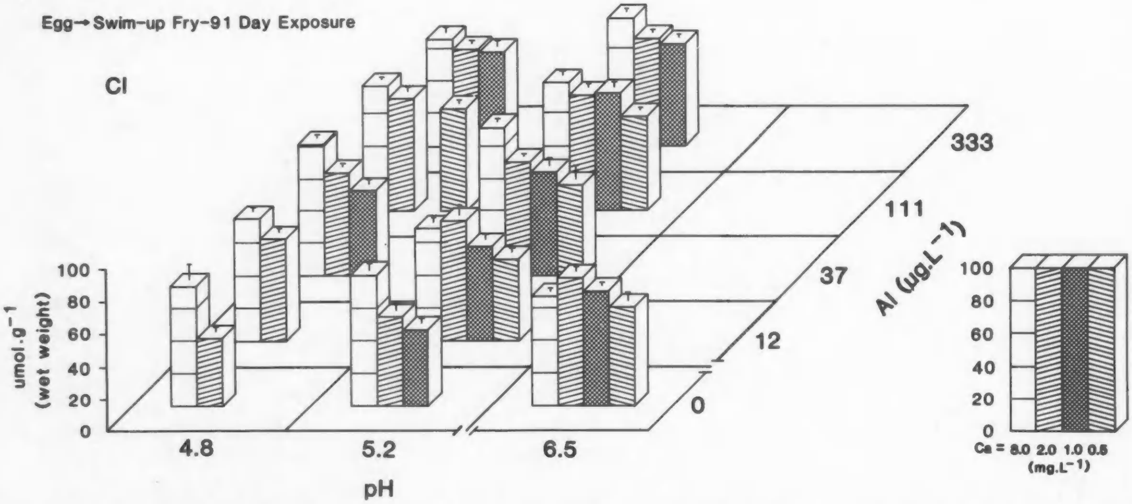

FIG. 6. Whole body concentrations of Cl in brook trout fry after 91 d continuous exposure to the designated matrix of pH, Ca, and Al. Other details as in legend of Fig. 4.

slight inhibitory effect, and Al a slight supportive effect (parabolic with log Al) which was greatest at 37 and 111 µg/L.

The positive influence of Ca was equally dominant for K (Fig. 7), the major intracellular electrolyte; here the relationship was strongest with log Ca. Again intermediate Al levels were slightly supportive, but pH had no significant effect (Fig. 2).

Not surprisingly, the greatest positive influence of Ca was on whole body Ca concentrations (Fig. 2, 8). Levels increased up to 3-fold in a step-wise fashion for any one cell over the range tested. Low pH had a small negative influence, and intermediate Al levels a small supportive effect.

The threshold for the supportive effect of water Ca on whole body Ca concentration at pH = 6.5 was between Ca = 0.5 and 1 mg/L, whereas under acidic conditions it lay between Ca = 2 and 8 mg/L (Fig. 8). Similar trends were seen for whole body Na (Fig. 5), Cl (Fig. 6), and K (Fig. 7), suggesting that the difference in threshold under acid conditions was a general phenomenon. None of the positive effects of Ca were due to expressing the data on a body weight basis, because Ca also positively influenced wet weight (Fig. 2, 4). Thus on a per fish basis, the effects were even greater.

In contrast to the other ions, whole body Mg was decreased by increasing water Ca (strongest with log Ca), an effect seen clearly in every cell of the matrix (Fig. 9). Acidity had a slight positive influence on Mg levels, while Al was without effect. Thus adverse conditions which tended to reduce the other ions and wet body weight also tended to raise whole body Mg concentrations.

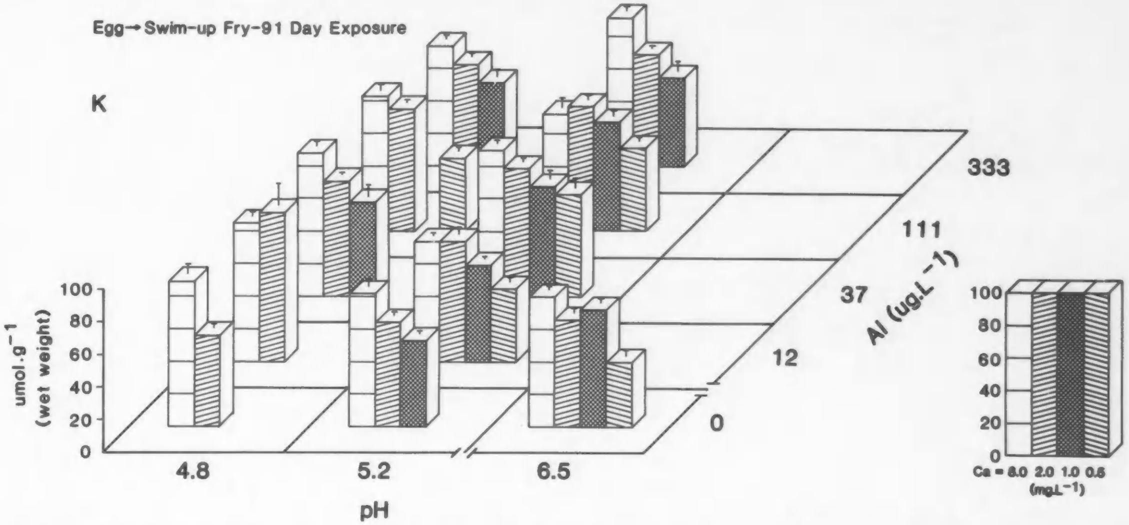

FIG. 7. Whole body concentrations of K in brook trout fry after 91 d continuous exposure to the designated matrix of pH, Ca, and Al. Other details as in legend of Fig. 4.

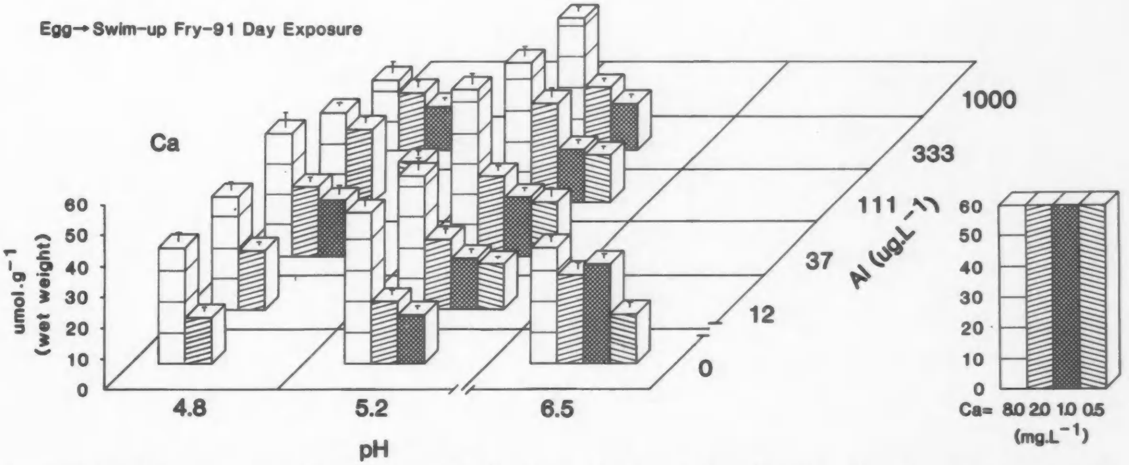

FIG. 8. Whole body concentrations of Ca in brook trout fry after 91 d continuous exposure to the designated matrix of pH, Ca, and Al. Other details as in legend of Fig. 4.

Aluminum Accumulation

Significant ΔAl was detected only at water Al = 111 and 333 μg/L, so was positively correlated with the water Al level Fig. 10). Over the matrix as a whole, the effects of Ca and acidity were small relative to those of water Al (Fig. 2). However, in those cells showing significant aluminum accumulation, both Ca and acidity had strong negative influences on ΔAl. For example, the peak accumulation of 9.4 μmol/g (at pH = 5.2, Ca = 1 mg/L, Al = 333 μg/L) was halved by either a reduction of pH to 4.8 at the same Ca, or by increasing Ca to 8 mg/L at the same pH.

Discussion

Electrolyte Concentrations and Changes during Development

Concentrations of Na, Cl, K, Ca, and Mg and their changes over time in developing brook trout embryos under control conditions, as determined by INAA in the present study (Fig. 1), were similar to those reported by Hayes et al. (1946) in developing *Salmo salar*. McCay et al. (1936) reported similar Ca concentration data in brook trout embryos. These early studies employed chemical analytical techniques. There was also good agreement with more recent atomic emission determinations of Na, K, Ca, and Mg in early life stages of other salmonid species (Zeitoun et al. 1976; Shen and Leatherland 1978a; Talbot et al. 1982). Direct comparison with the most detailed study, that of Peterson and Martin-Robichaud (1986) on Na, K, Ca, and Mg changes (by ion chromatography) in developing *Salmo salar*, is difficult because of the different basis for data expression used in their study. Nevertheless, the two studies appear in broad qualitative agreement.

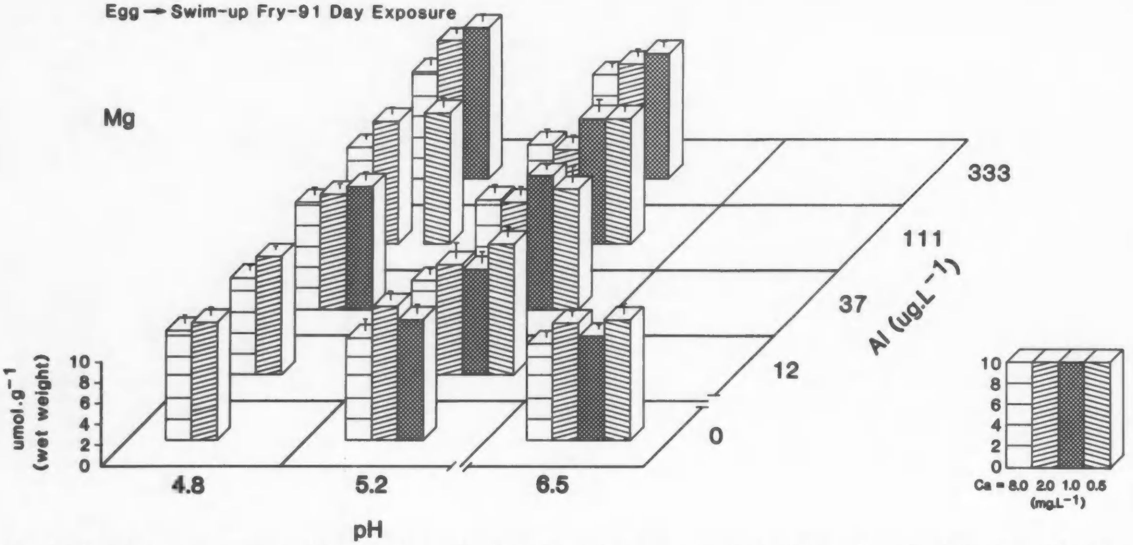

FIG. 9. Whole body concentrations of Mg in brook trout fry after 91 d continuous exposure to the designated matrix of pH, Ca, and Al. Other details as in legend of Fig. 4.

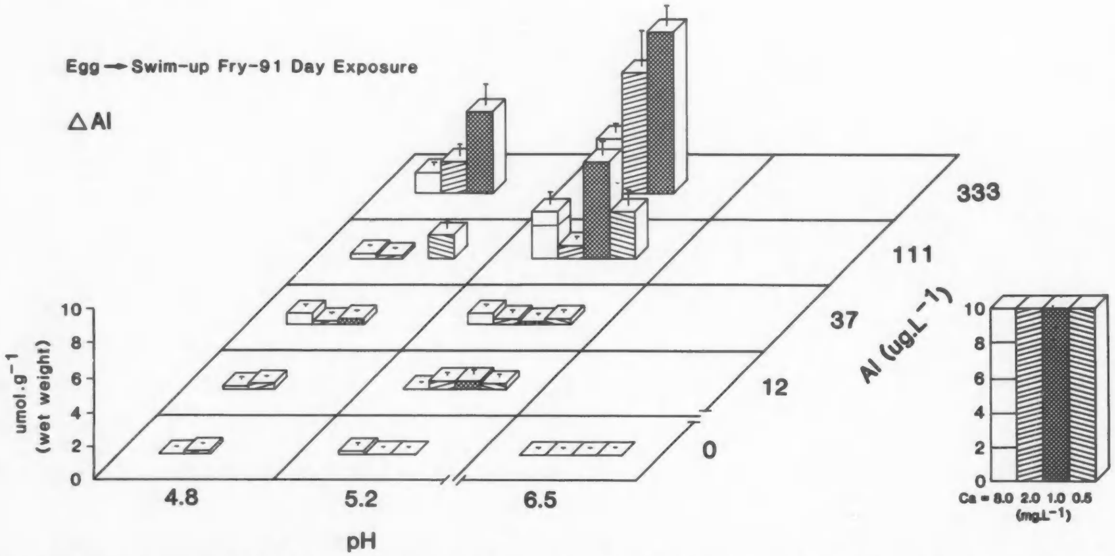

FIG. 10. Whole body accumulation of aluminum (ΔAl) in brook trout fry after 91 d continuous exposure to the designated matrix of pH, Ca, and Al. Other details as in legend of Fig. 4.

A finding common to all these studies is that development from fertilization through yolk-sac resorption involves marked net accumulation of electrolytes by the embryo (cf. Fig. 1). The detailed analyses of Hayes et al. (1946), Zeitoun et al. (1976), Rombough and Garside (1984), and Peterson and Martin–Robichaud (1986) indicate that the external environment is the source of more than half the Na and Ca uptake, and at least in three of the studies, a smaller fraction of the K uptake. Magnesium is the only ion present in the yolk in clear excess of the requirements of the embryo; it therefore decreases in concentration during development (cf. Fig. 1). The major routes of ion uptake are thought to occur via rudimentary "chloride cells" which occur on the yolk-sac epithelium and skin, as well as on the developing gills (Shen and Leatherland 1978b; Hwang and Hirano 1985). Net accumulation will represent the difference between active uptake from and diffusive loss to the dilute external environment. Exogenous feeding may also play a role, especially after swim-up (Talbot et al. 1982). However, to date it has not been possible to quantify the contribution of this route to net ion accumulation.

Effects of 91 D Exposure

The most important finding of the present study is that water Ca level, rather than pH or Al, was the major determinant of net electrolyte accumulation in fish which have been raised continuously from fertilization through swim-up in an extensive pH/Ca/Al matrix (Fig. 2). The effects were most dramatic for Ca and Na, where water Ca explained more than 50% of the overall variance; for Cl and K, Ca accounted for more than 35% of the variance. This does not mean that acidity and Al were without toxic effect. Indeed acidity was the major determinant of mortality in this exposure prior to hatching, and Al was the major determinant after hatching (Ingersoll et al. 1990). While Ca was positively related to survival over both periods, its influence was less than those of the other two factors. In other words, the toxic effects of pH and Al eliminated all of the fish in many of the exposure cells and depleted other cells prior to the sample time of this experiment. What remained were survivors where the positive influence of Ca was clearly the dominant sublethal effect.

This large positive influence of Ca on whole body Na, Cl, K, and Ca levels, the much smaller positive influence of Al, and the negative influence of acidity could result from effects on developmental rate and/or on ion exchange rates. Body weight, either wet or dry, is not a good index of developmental rate for these early life stages because it tends to decrease as development proceeds under control conditions (Fig. 1; also Geen et al. 1985). Furthermore it may be affected in a complex fashion by adverse conditions, as indicated by the biphasic pattern in dry weight with pH, combined with the negative influence of acidity and positive influence of Ca on wet weight (Fig. 4). When taken together with the temporal changes in Fig. 1 and the observations of Ingersoll et al. (1990) that low pH delayed hatching, yolk-sac resorption, swim-up, and feeding, these data suggest that the body weight changes resulted from two opposing influences. Unfavourable conditions tended to delay development, thereby reducing the decrease in weight (especially dry weight) due to yolk-sac resorption. At the same time, these conditions tended to increase metabolic costs and reduce food intake, thereby accelerating metabolic weight loss and decreasing embryo growth. Thus, depending on their severity, adverse conditions which delay development may either retard or accelerate the normal weight loss associated with ontogeny.

However, Mg present in high concentration in the yolk but low concentration in the embryo (Hayes et al. 1946; Peterson and Martin–Robichaud 1986), appears to be a useful marker for developmental rate (Fig. 1). Whole body Mg concentrations were clearly lowered by increasing Ca and slightly raised by increasing acidity, in contrast to the trends with all other ions (Fig. 9). This suggests that development was delayed by low water Ca and low pH. Many other studies have shown that low pH, either alone or in combination with Al, inhibits development in early life stages of salmonids (eg. Geen et al 1985; Peterson and Martin–Robichaud 1986; Hunn et al 1987; Gunn and Noakes, 1987), but the marked effect of low water Ca has not been documented previously.

On the basis of the present data alone, it is not possible to conclude whether effects of Ca, Al, and pH on whole body ions were due to altered ion exchange rates or to altered developmental rates, or both. Indeed, developmental rate may either limit or be limited by the rate of net electrolyte accumulation. However, the companion study which examines age-specific challenges in the matrix (Wood et al. 1990) indicates that both effects occur and, at least in the yolk-sac stage, that pronounced influences on whole body ion accumulation can be demonstrated in the absence of developmental effects. Peterson and Martin–Robichaud (1986) also documented an inhibition of ion accumulation which was both separate from and additional to inhibited development in *Salmo salar* embryos exposed to low pH alone for 135 d.

The negative effect of low pH on body ion concentrations has been repeatedly demonstrated in adult fish, where it results from both inhibition of active uptake, probably through the "chloride cells," and stimulation of diffusive loss, probably through the paracellular channels of the gills (cf. Wood 1989). Similar explanations probably apply to the transporting epithelia (vitelline membrane, developing gills) of the embryo. In the adult, the protective effect of water Ca is largely due to its action in curtailing diffusive losses by reducing branchial permeability (Wood 1989). This is probably also the basis of its present dramatic action on whole body ion levels in embryos. The particularly marked effect of water Ca on whole body Ca levels (Fig. 8) may result from an additional factor, the pronounced concentration-dependence of branchial Ca uptake which has been documented in adult fish (Mayer–Gostan et al. 1983; Perry and Wood 1985). The small but significant "parabolic" effect of Al on most whole body ions is considered in greater detail by Wood et al. (1990). In brief, it can be interpreted as a protective action at intermediate levels due to the ability of Al to either substitute for Ca in stabilizing membrane permeability or to induce proliferation of "chloride cells." At higher levels, this protection disappears as Al itself becomes toxic to ionoregulation.

Protective effects of Al were seen at water Al levels below which significant ΔAl accumulation was detected (Fig. 10). Accumulation occurred in exposure cells (pH = 5.2, Al = 111 and 333 μg/L; pH = 4.8, Al = 333 μg/L) where Al was close to its solubility limit (Johnson et al. 1981). Some or all of the ΔAl may represent an adherence to the external surface of the embryos. This could result from precipitation in the more alkaline micro-environment of the fish and/or binding to anionic organic ligands on the surface. The inhibitory effect of lower pH and higher Ca on ΔAl accumulation could result from H^+ and Ca^{2+} competition for binding sites, the change in relative speciation from a predominance of cationic hydroxides at higher pH to free trivalent Al^{3+} at lower pH, and the greater absolute solubility of Al at lower pH. Cleveland et al. (1986) found a similar inhibition of Al accumulation by lower pH (4.5 vs. 5.5) in early life stages of brook trout exposed to Al = 300 μg/L at Ca = 3 mg/L for 30 d; absolute ΔAl levels were similar in the two studies.

Environmental Significance

The protective action of Ca against the adverse effects of low pH and/or high Al on ionoregulation in adults and on survival in early life stages is well known (eg. Howells et al. 1983; Wood 1989; McDonald et al. 1989). However, the present sublethal effects on mineralization are the largest yet documented. Furthermore, this appears to be the first demonstration that water Ca has a critical influence on net ion accumulation even at pH = 6.5, in the absence of Al. The hatching to swim-up period is a time of intense mineralization; water Ca level will have a marked effect under neutral conditions, and an even greater influence under low pH and/or high Al conditions. For example, a fry raised for the first 91 d of its life at Ca = 0.5 mg/L (at pH = 6.5) would have only about 75% of the Na and Cl concentration, and only about 50% of the K and Ca concentration of one raised at Ca = 1, 2 or 8 mg/L. If the pH were

4.8 for the first 91 d, even at Ca = 2 mg/L, these values would be reduced to about 55% for Na and Cl, and 40% for Ca. The long-term survival prospects of such fish are probably poor. However, Ca = 8 mg/L affords almost complete protection, even at pH = 4.8.

This analysis emphasizes that the threshold for the beneficial effects of Ca, which is between 0.5 and 1 mg/L at pH = 6.5, is shifted to a higher Ca level, between 2 and 8 mg/L, under low pH. Thus natural soft waters which have sufficient Ca to support healthy fish development at circumneutral pH may lose this capacity if acidified. A Ca level of 8 mg/L is at the upper end of the softwater range, and probably unlikely to occur in most natural acid stress situations. This conclusion as to the importance of Ca, based on whole body ion analysis, is in general agreement with those of Wright and Snekvik (1978) and Howells et al. (1983) based on field surveys of acidified lakes. These surveys also indicated that Al was not a major determinant of fishery status in acidified lakes, which again is in accord with the present data.

One important reservation about the environmental significance of the present study must be noted. Here, fish were exposed continuously to toxic conditions from fertilization through swim-up. However, in the wild, brook trout may select areas of groundwater seepage for spawning, and avoid areas of low pH (Johnson and Webster 1977; Gunn 1986). The water in the redd may therefore be relatively alkaline, higher in Ca, and lower in Al than that in the overlying stream. Exposure to toxic conditions may only occur when the alevins emerge from the substrate to commence feeding (Trojnar 1977). The experiments of the following paper were designed to evaluate this situation (Wood et al. 1990).

Acknowledgements

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References

Cleveland, L., E. E. Little, S. J. Hamilton, D. R. Buckler, and J. B. Hunn. 1986. Interactive toxicity of aluminum and acidity to early life stages of brook trout. Trans. Am. Fish. Soc. 115: 610–620.

Cunningham, G. L., and B. J. Shuter. 1986. Interaction of low pH and starvation on body weight and composition of young-of-year smallmouth bass (*Micropterus dolomieui*). Can. J. Fish. Aquat. Sci. 43: 869–876.

Desote, D., R. Gubels, and J. Hoste. 1972. Growth and decay of radioactivity during and after irradiation, p. 123–159. Neutron Activation Analysis, Vol. 34 of Chemical Analysis. Wiley-Interscience, Toronto, Ont.

Fraser, G. A., and H. H. Harvey. 1984. Effects of environmental pH on the ionic composition of white sucker (*Catostomus commersoni*) and pumpkin-seed (*Lepomis gibbosus*). Can. J. Zool. 62: 249–259.

Geen, G. H., J. D. Neilsen and M. Bradford. 1985. Effects of pH on the early development and growth and otolith microstructure of chinook salmon, *Oncorhynchus tshawytscha*. Can. J. Zool. 63: 22–27.

Gunn, J. M. 1986. Behaviour and ecology of salmonid fishes exposed to episodic pH depressions. Envir. Biol. Fish. 17: 241–252.

Gunn, J. M., and D. L. G. Noakes. 1987. Latent effects of pulse exposure to aluminum and low pH on size, ionic composition, and feeding efficiency of lake trout (*Salvelinus namaycush*) alevins. Can. J. Fish. Aquat. Sci. 44: 1418–1424.

Haines, T. A. 1981. Acidic precipitation and its consequences for aquatic ecosystems: a review. Trans. Am. Fish. Soc. 110: 669–707.

Hayes, F. R., D. A. Darcy, and C. M. Sullivan. 1946. Changes in the inorganic constituents of developing salmon eggs. J. Biol. Chem. 163: 621–633.

Heydorn, K. 1984. Neutron Activation Analysis for Clinical Trace Element Research. C.R.C. Press, Baton Rouge, LA.

Howells, G. D. 1984. Fishery decline: mechanisms and predictions. Philos. Trans. R. Soc. Lond. B305: 529–547.

Howells, G. D., D. J. A. Brown, and K. Sadler. 1983. Effects of acidity, calcium and aluminium on fish survival and productivity — a review. J. Sci. Food Agric. 34: 559–570.

Hunn, J. B., C. L. Cleveland, and E. E. Little. 1987. Influence of pH and aluminum on developing brook trout in low calcium water. Environ. Pollut. 43: 63–73.

Hwang, P. P. and P. Hirano. 1985. Effects of environmental salinity on intercellular organization and junctional structure of chloride cells in early stages of teleost development. J. Exp. Zool. 236: 115—126.

Ingersoll, C. G., D. Gulley, D. R. Mount, T. W. La Point, and H. L. Bergman. 1990. Effects of pH, aluminum, and calcium on survival and growth of eggs and fry of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 1580–1592.

Johnson, N. M., C. T. Driscoll, J. S. Eaton, G. E. Likens, and W. H. McDowell. 1981. ''Acid Rain,'' dissolved aluminum, and chemical weathering at the Hubbard Brook Experimental Forest, New Hampshire. Geochim. Cosmochim. Acta 45: 1421–1438.

Johnson, D. W., and D. A. Webster. 1977. Avoidance of low pH in selection of spawning sites by brook trout (*Salvelinus fontinalis*). J. Fish. Res. Board Can. 34: 2215–2218.

Leivestad, H. 1982. Physiological effects of acid stress on fish, p. 157–164. *In* R.E. Johnson [ed.] Acid Rain/Fisheries. Am. Fish. Soc. Bethesda, MD.

Mayer–Gostan, N., M. Bornancin, G. DeRenzis, R. Naon, J. A. Yee, R. L. Shew, and P. K. T. Pang. 1983. Extraintestinal calcium uptake in the killifish, *Fundulus heteroclitus* J. Exp. Zool. 227: 329–338.

McCay, C. M., A. V. Tunison, M. Crowell, and H. Paul. 1936. The calcium and phosphorus content of the body of the brook trout in relation to age, growth, and food. J. Biol. Chem. 114: 259–263.

McCormick, J. H., K. E. F. Hokanson, and B. R. Jones. 1972. Effects of temperature on growth and survival of young brook trout, *Salvelinus fontinalis*. J. Fish. Res. Board Can. 29: 1107–1112.

McDonald, D. G., J. P. Reader, and T. K. R. Dalziel. 1989. The combined effects of pH and trace metals on fish ionoregulation, p. 221–242. *In* R. Morris, D. J. A. Brown, E. W. Taylor, and J. A. Brown [ed.] Acid Toxicity and Aquatic Animals, Society for Experimental Biology Seminar Series. Cambridge University Press, UK.

Perry, S. F., and C. M. Wood. 1985. Kinetics of branchial calcium uptake in the rainbow trout: effects of acclimation to various external calcium levels. J. Exp. Biol. 116: 411–433.

Peterson, R. H., P. G. Daye, G. L. Lacroix, and E. T. Garside. 1982. Reproduction in fish experiencing acid and metal stress, p. 177–196. *In* R. E. Johnson [ed.] Acid Rain/Fisheries Am. Fish. Soc. Bethesda, MD.

Peterson, R. H., and D. J. Martin–Robichaud. 1986. Growth and major inorganic cation budgets of Atlantic salmon alevins at three ambient acidities. Trans. Am. Fish. Soc. 115: 220–226.

Rombough, P. J., and E. T. Garside. 1984. Disturbed ion balance in alevins of Atlantic salmon *Salmo salar* chronically exposed to sublethal concentrations of cadmium. Can. J. Zool. 62: 1443–1450.

Shearer, K. D. 1984. Changes in elemental composition of hatchery-reared rainbow trout, *Salmo gairdneri*, associated with growth and reproduction. Can. J. Fish. Aquat. Sci. 41: 1592–1600.

Shen, A. C. Y., and J. F. Leatherland. 1978a. Effect of ambient salinity on ionic and osmotic regulation of eggs, larvae, and alevins of rainbow trout (*Salmo gairdneri*). Can. J. Zool. 56: 571–577.

1978b. Structure of the yolksac epithelium and gills in the early developmental stages of rainbow trout (*Salmo gairdneri*) maintained in different ambient salinities. Environ. Biol. Fishes 3: 345–354.

Talbot, C., F. B. Eddy, and J. Johnston. 1982. Osmoregulation in salmon and sea trout alevins. J. Exp. Biol. 101: 61–70.

Trojnar, J. R. 1977. Egg hatchability and tolerance of brook trout (*Salvelinus fontinalis*) fry at low pH. J. Fish. Res. Board Can. 34: 574–579.

Wood, C. M. 1989. The physiological problems of fish in acid waters, p. 125–152. *In* R. Morris, D. J. A. Brown, E. W. Taylor, and J. A. Brown [ed.] Acid Toxicity and Aquatic Animals, Society for Experimental Biology Seminar Series. Cambridge University Press, UK.

WOOD, C. M., D. G. MCDONALD, C. E. BOOTH, B. P. SIMONS, C. G. INGERSOLL, AND H. L. BERGMAN. 1988. Physiological evidence of acclimation to acid/aluminum stress in adult brook trout (*Salvelinus fontinalis*). 1. Blood composition and net sodium fluxes. Can. J. Fish. Aquat. Sci. 45: 1587–1596.

WOOD, C. M., D. G. MCDONALD, C. G. INGERSOLL, D. R. MOUNT, O. E. JOHANNSSON, S. LANDSBERGER, AND H. L. BERGMAN. 1990. Whole body ions of brook trout (*Salvelinus fontinalis*) alevins: responses of yolk-sac and swim-up stages to water acidity, calcium, and aluminum, and recovery effects. Can. J. Fish. Aquat. Sci. 47: 1604–1615.

WRIGHT, R. F., AND E. SNEKVIK. 1978. Acid precipitation: chemistry and fish populations in 700 lakes in southernmost Norway. Ver. Int. Vere. Limnol. Biol. 20: 765–775.

I. H. ZEITOUN, D. E. ULLREY, W. G. BERGEN, AND W. T. MAGEE. 1976. Mineral metabolism during the ontogenesis of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 33: 2587–2591.

Whole Body Ions of Brook Trout (*Salvelinus fontinalis*) Alevins: Responses of Yolk-Sac and Swim-Up Stages to Water Acidity, Calcium, and Aluminum, and Recovery Effects

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Water pH, rather than Ca or Al, was the most important factor affecting whole body ions in yolk-sac or swim-up fry exposed to a matrix of pH (6.5–4.0), Ca (0.5–8 mg/L), and Al (0–1000 μg/L). Fry were raised from fertilization (day 0) in flowing soft water (pH = 6.5, Ca = 2 mg/L, Al = 0 μg/L), exposed to pH/Ca/Al on day 49 (yolk-sac, 2 d post-hatch) or day 70 (swim-up) for 21 d, and then allowed to recover a further 20 d. Yolk-sac fry were extremely resistant at pH ≥ 4.4; developmental effects, as indicated by body weight and Mg, were negligible. However whole body Na, Cl, K, and Ca were depressed by low pH, while water Ca was protective. Aluminum (37–111 μg/L) raised most ions above control values, while higher Al lowered them. Swim-up fry were more sensitive, showing pronounced developmental inhibition (lower weight, higher Mg) under adverse conditions; mortality continued during recovery. Low pH was again the dominant influence on body ions, water Ca was protective, while Al (12–111 μg/L) was only protective and not stimulatory. These effects persisted significantly; indeed responses in body Ca were larger after recovery than after the exposure itself. In the field, emergence from the redd into ambient acidic water is probably the critical stage. Water pH will be the principal determinant of whole body ions in alevins surviving this emergence, in contrast to fry exposed continuously from fertilization.

Le pH de l'eau plutôt que la conc. en Ca ou en Al, a constitué le plus important facteur à agir sur l'équilibre électrolytique d'alevins au stade du sac vitellin et au stade de la remontée à la nage et qui étaient exposés à des combinaisons de pH (6,5–4,0), de conc. en Ca (0,5–8 mg/L) et d'Al (0–1000 μg/L). Les alevins ont été gardés dès la fécondation (jour 0) dans une eau douce en circulation (pH = 6,5, conc. Ca = 2 mg/L, conc. Al = 0 μg/L), et exposés aux combinaisons de pH/Ca/Al au jour 49 (sac vitellin, 2 jours après l'éclosion) ou au jour 70 (remontée à la nage) pendant 21 jours; une période de récupération de 20 jours a été accordée. Les alevins au stade du sac vitellin étaient extrêmement résistants à pH 4,4 et plus; les effets sur le développement mesurés par la masse corporelle et la teneur en Mg étaient négligeables. Cependant, l'équilibre électrolytique en Na, Cl, K et Ca a été déprimé à pH acide alors que la concentration de Ca dans l'eau conférait une protection. La conc. en Al à 37–111 μg/L a fait passer la conc. de la plupart des ions à des valeurs supérieures aux valeurs de référence alors que les conc. élevées en Al ont eu l'effet contraire. Les alevins qui remontaient à la nage étaient plus sensibles que les autres et manifestaient une profonde inhibition de leur développement (poids inférieur, conc. en Mg supérieure) dans des conditions adverses; il y a eu de la mortalité pendant la période de récupération. Les bas pH sont demeurés l'influence dominante sur l'équilibre électrolytique, la conc. en Ca dans l'eau assurait une protection alors que les conc. en Al (12–111 μg/L) avaient seulement un effet de protection, mais pas de stimulation. Ces effets ont persisté de manière significative; en effet, la réaction des organismes mesurée en termes de Ca était plus importante après la période de récupération qu'après l'exposition elle-même. Sur le terrain, le

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passage du nid de fraie à l'eau acide ambiante constitue probablement l'étape critique. Le pH de l'eau constitue le principal déterminant de l'équilibre électrolytique chez les alevins qui survivent à cette émergence, contrairement aux alevins exposés continuellement depuis la fécondation.

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In the preceding study, water calcium concentration (Ca) was the principal determinant of whole body ions in surviving brook trout fry which had been continuously exposed to a pH/Ca/Al matrix for 91 d from fertilization through swim-up (Wood et al. 1990). Water pH had a negative effect on most whole body ions, but its influence was small relative to that of Ca while Al had a slight positive influence at intermediate concentrations. While these findings are relevant to the field situation of salmonid species such as the lake trout (*Salvelinus namaycush*; Gunn and Keller 1984a) and Atlantic salmon (*Salmo salar*; Lacroix 1985) where the earliest life stages may be exposed to such conditions, there is some question as to their relevance to the natural reproduction of brook trout (*Salvelinus fontinalis*). Adult brook trout may select regions of groundwater seepage, especially alkaline upwellings, in which to build their redds, and actively avoid areas of low pH when spawning (Webster and Eiriksdottir 1976; Johnson and Webster 1977). As Trojnar (1977) and Gunn (1986) have pointed out, this strategy may effectively insulate the earliest developmental stages from adverse conditions. Instead, toxicity may occur primarily when the older alevins (yolk-sac or swim-up fry) emerge from the gravel into overlying softer, more acidic, Al-contaminated water, especially during episodic acid surges accompanying snow-melt in early spring.

The principal goal of the present study was to determine whether similar or different effects on whole body ions occurred when brook trout were exposed to pH/Ca/Al conditions only in the yolk-sac or swim-up fry stages, rather than continuously from fertilization to swim-up. The exposures were performed as part of the toxicological studies reported by Ingersoll et al. (1990a). To facilitate comparison with the 91 d continuous exposure study (Wood et al. 1990), the experiments were performed simultaneously, and the fry stocks, exposure matrix, and analytical techniques were all identical. A second objective was to compare the sensitivity and responses of yolk-sac vs. swim-up fry. The relative importance of the gills as an ionoregulatory and respiratory site should be greater in swim-up fry, and the importance of the vitelline membrane and skin for these functions correspondingly reduced. A final goal was to examine recovery effects on whole body ions and Al accumulation in survivors, for by definition episodic acid surge events are short-lived, generally lasting a few days to a few weeks (Christophersen et al. 1984; Marmorek et al. 1985).

Materials and Methods

Ingersoll et al. (1990a) and Wood et al. (1990) describe details of the fish, the experimental regimes, the water chemistry, the sampling procedures, the instrumental neutron activation analysis (INAA) technique for whole body ions and Al, and the statistical procedures used in analyzing the data. The 84 cell matrix of pH (6.5, 5.2, 4.8, 4.4, 4.0), Ca (0.5, 1, 2, 8 mg/L), and Al (0, 12, 37, 111, 333, 1000 μg/L) was the same as that used in the 91 d exposures. In the yolk-sac fry experiments, cells at Ca = 1 and 8 mg/L and Al = 12 μg/L were not sampled for INAA. In the swim-up fry experiments, cells at Ca = 1 mg/L were not sampled.

In brief, for the studies which constitute the focus of the present paper, brook trout were raised from fertilization under control conditions (pH = 6.5, Ca = 2 mg/L, Al = 0 μg/L) in flowing artificial soft water (Na≃4, Cl≃8 mg/L, 10.9°C). Control conditions were maintained until the fish were exposed to the pH/Al/Ca matrix. After exposure, fish were returned to control conditions for the recovery period. Typically, 8–10 survivors were preserved for INAA from each cell which was sampled, though this was reduced in cases where mortality had rendered this number unavailable. The smallest numbers taken were four per cell at the end of the challenges, and three per cell at the end of the recoveries. Each fish was analyzed by INAA for whole body concentrations of Na, Cl, K, Ca, Mg, and aluminum accumulation (ΔA1) as described by Wood et al. (1990). In both yolk-sac and swim-up series, several cells were sacrificed for other purposes, and therefore could not be used for INAA.

The yolk-sac fry challenge started on day 49 post-fertilization, which was about 2 d after the median time of hatch. Twenty-five yolk-sac fry were transferred into each cell of the matrix for 21 d. After sampling on day 70, the remaining alevins were returned to the control condition for a further 20 d. Recovery samples were taken on day 90. In these yolk-sac fry experiments, only the surviving fish challenged at Ca levels of 0.5 and 2 mg/L were sampled for INAA, and fish at Al = 12 μg/L were omitted.

The swim-up fry challenge started on day 70, by which time exogenous feeding had been occurring for 10–15 d (though unquantifiable) and the yolk-sac appeared to be about 50% resorbed. Again, 25 fish were transferred into each cell of the matrix for 21 d. After sampling on day 91, the remaining fry were returned to the control condition for 20 d. Recovery samples were taken on day 111. In these swim-up fry experiments, the surviving fish challenged at Ca levels of 0.5, 2, and 8 mg/L were sampled for INAA.

Results

Yolk-Sac Fry — Relative Influence of pH, Ca, and Al

Figure 1 summarizes the results of the multiple regression analyses for the major measured parameters at the end of the 21 d exposure, and after a further 20 d recovery under control conditions. Except for the very small effect of water pH on whole body Mg after recovery (Fig. 1B), all factors significant by this analysis were also significant by factorial analysis of variance. The proportion of overall variance explained by the three measured parameters (Ca, Al, and pH) was much greater immediately after exposure (Fig. 1A) than after 20 d recovery (Fig. 1B). Nevertheless, small effects, particularly of pH, persisted. After exposure, pH was the dominant influence on whole body Na and Cl, followed by Ca and then Al, the three together accounting for approximately 60% of the variance

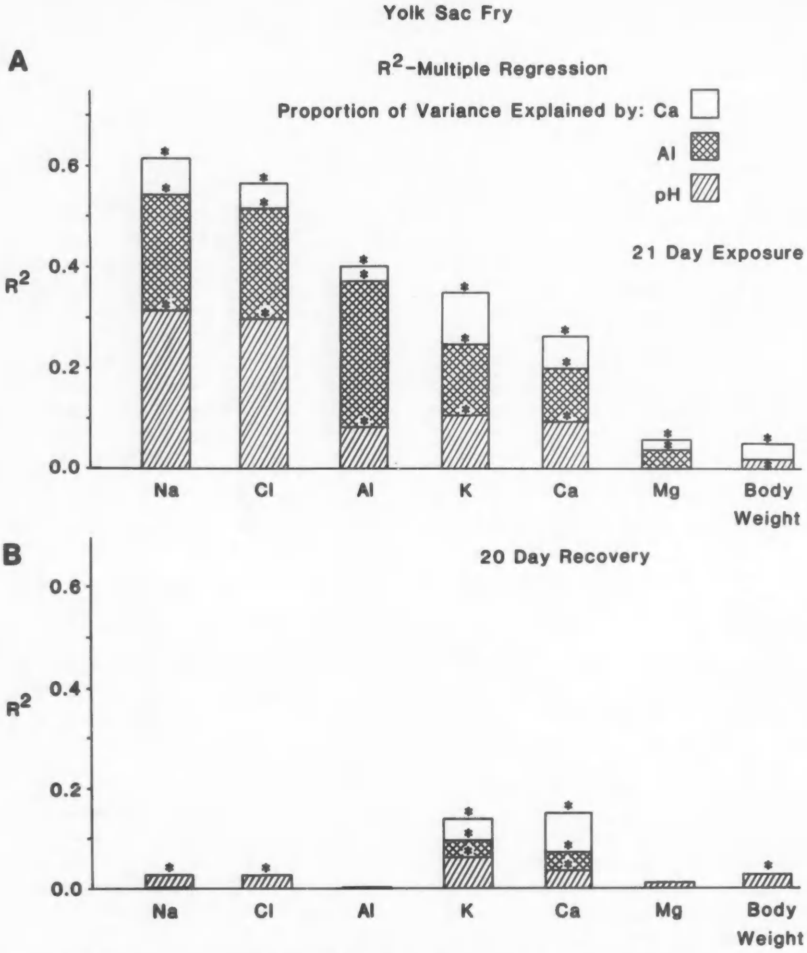

FIG. 1. Total variance explained (R^2) and proportion attributable to each factor (Ca, Al, pH of the exposure matrix), as determined by stepwise multiple regression analysis, for various whole body parameters in yolk-sac fry of brook trout. Results are shown for fry after 21 d exposure and following 20 d recovery under control conditions (pH = 6.5, Ca = 2 mg/L, Al = 0 μg/L). Asterisks indicate relationships which were also significant by factorial analysis of variance.

(Fig. 1A). For whole body K and Ca, the three factors accounted for approximately equal, though much smaller, percentages of the variance. Not surprisingly, water Al was the dominant influence on whole body ΔAl, an effect which completely disappeared after 20 d recovery (Fig. 1B).

Yolk-Sac Fry — Mortality and Body Weights

In terms of survival, yolk-sac fry were extremely resistant to low pH and Al, except at pH = 4.0, where mortality was virtually complete (cf. Ingersoll et al. 1990a). At the two Ca levels sampled for INAA (0.5 and 2 mg/L), survival was 80–100% in most cells at pH = 4.4 and above. The only exceptions were pH = 4.4 at Al = 0 or 1000 μg/L), where survival was 30–45%; intermediate levels of Al (37–333 μg/L) were protective at pH = 4.4. Ca had no significant effect. During recovery, survival was again high (80–100%), and there were no significant effects of the previous pH, Ca, or Al levels during challenge.

Body weight, either wet or dry, was similarly unresponsive to pH, Ca, or Al, during both challenge and recovery. Multiple regression analyses detected significant positive effects on wet weight of pH and Ca during the challenge and of pH alone during recovery. However the R^2 values were so small (<0.04) as to be of doubtful biological importance. There were no effects on dry weight.

Yolk-Sac Fry — Whole Body Electrolytes after Challenge

Despite the insensitivity of mortality and body weight to the experimental conditions in yolk-sac fry, INAA revealed marked sublethal effects on all whole body ions except Mg.

After 21 d exposure, pH was the dominant influence on the concentrations of the two major extracellular electrolytes, Na

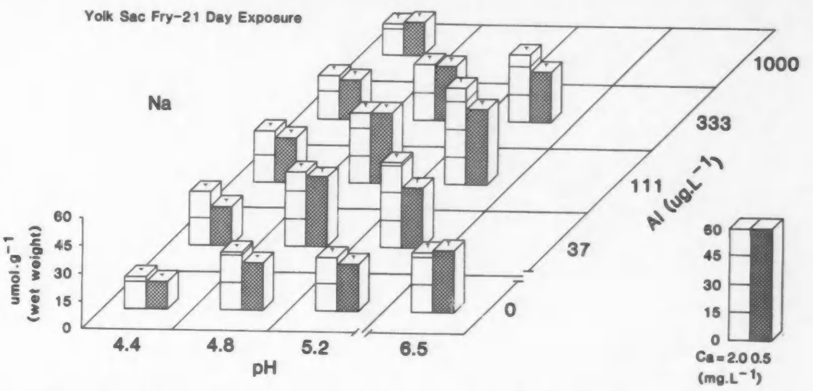

FIG. 2. Whole body concentrations of Na in yolk-sac fry of brook trout after 21 d exposure to a matrix of pH, Ca, and Al. Means ± 1 SEM for $n=8$–10 fish in most cells, falling as low as four in cells with high mortality.

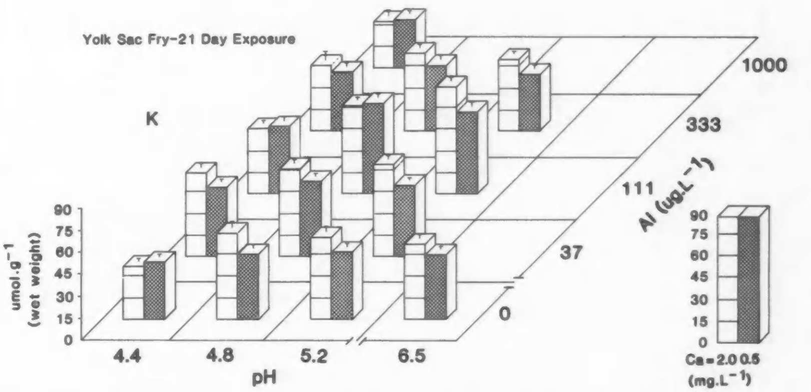

FIG. 3. Whole body concentrations of K in yolk-sac fry of brook trout after 21 d exposure to a matrix of pH, Ca, and Al. Other details as in legend of Fig. 2.

and Cl, accounting for about 30% of the overall variance (Fig. 1A). Only the Na data are shown (Fig. 2), for all trends with Cl were very similar, though absolute concentrations were almost twice as high at this stage, day 70 (cf. figure 1 of Wood et al. 1990). In the absence of Al, whole body Na and Cl were depressed in a linear fashion by low pH, reaching only half the control levels at pH = 4.4 (Fig. 2). At all acidic pH's, intermediate levels of Al exerted pronounced protective/stimulatory effects on Na and Cl concentrations. These effects were greatest at Al = 37 and 111 μg/L, and were best described by parabolic relationships with log Al, which explained a further 20% of the overall variance (Fig. 1A). Al not only protected the fish against the inhibitory effects of low pH, but actually raised whole body Na and Cl levels up to 65% *above* the control values. These stimulatory effects were seen most clearly at pH = 4.8 and 5.2 with Al = 37 and 111 μg/L (Fig. 2). Exposure to higher levels of water Ca (2 vs. 0.5 mg/L) also exerted a small positive influence in most cells.

Whole body K was also strongly affected by the exposures (Fig. 3), being lowered by acidity, raised by Ca and intermediate Al levels (37–111 μg/L), and slightly depressed by the highest Al levels (333–1000 μg/L). Each factor accounted for about 10% of the total variance (Fig. 1A). As for Na and Cl, K was significantly elevated *above* control levels by the presence of Al in several cells at pH 4.8 and 5.2; the absolute increases were approximately 30%. The overall effect was best described by a parabolic relationship with Al.

Whole body concentrations of Ca were much lower than those of K, but responded in a generally similar fashion after 21 d challenge (Fig. 1A, 4). Thus Ca was depressed in a linear fashion by low pH, increased by water Ca, and affected by Al in a parabolic manner. The highest Al levels tended to decrease whole body Ca, while intermediate levels were clearly protective. However, the presence of Al raised Ca above the control level in only one cell, a less pronounced effect than seen for Na, Cl, and K.

In contrast to the other ions, Mg was largely unaffected by the exposure conditions. There were weak negative influences of Al and Ca, but together these accounted for less than 6% of the overall variance; pH had no significant effect (Fig. 1A).

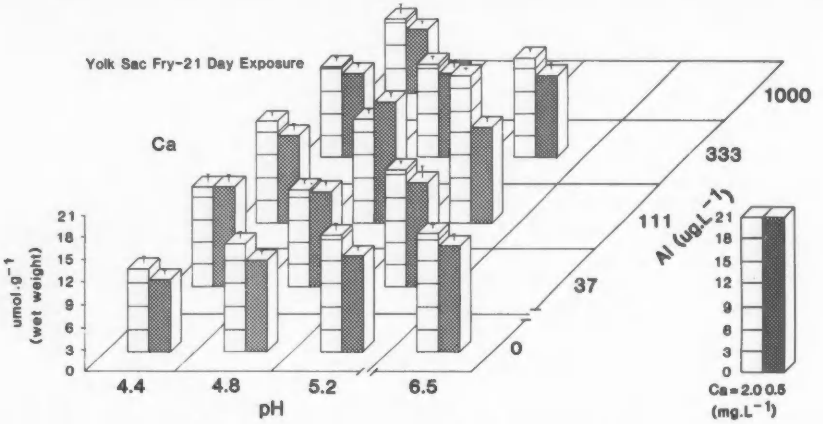

FIG. 4. Whole body concentrations of Ca in yolk-sac fry of brook trout after 21 d exposure to a matrix of pH, Ca, and Al. Other details as in legend of Fig. 2.

TABLE 1. A comparison of whole body ion levels (μmol/g wet weight) in selected cells (Ca = 2 mg/L) of the yolk-sac fry challenge experiment at the end of 21 d exposure, and after 20 d of recovery under control conditions (pH = 6.5, Ca = 2 mg/L, Al = 0 μg/L). Means ± 1 SEM (*N*). * = $p \leqslant 0.05$ relative to comparable control value (pH = 6.5, Al = 0 μg/L) at this time; † = $p \leqslant 0.05$ relative to comparable value for this same treatment at end of 21 d exposure.

		21 d exposure (day 70)			20 d recovery (day 90)		
pH	Al	Na	K	Ca	Na	K	Ca
6.5	0	32.7 ±1.6 (10)	52.5 ±3.2 (10)	16.0 ±0.7 (10)	$51.0^{\dagger}$ ±4.1 (9)	$72.3^{\dagger}$ ±6.1 (9)	$24.1^{\dagger}$ ±1.5 (9)
5.2	111	54.4^{*} ±2.6 (9)	73.9^{*} ±2.7 (9)	20.1^{*} ±0.3 (9)	52.5 ±7.1 (5)	69.7 ±2.4 (5)	$25.8^{\dagger}$ ±1.7 (5)
4.4	0	17.6^{*} ±0.4 (10)	36.9^{*} ±1.6 (10)	$10.9^{\dagger}$ ±0.8 (10)	$34.0^{\dagger,*}$ ±6.8 (6)	$45.1^{\dagger,*}$ ±4.0 (6)	$17.3^{\dagger,*}$ ±1.7 (6)

Yolk-Sac Fry — Whole Body Electrolytes after Recovery

Twenty days of recovery under control conditions (by which time the fry were in the swim-up stage) removed most of the effects of pH, Al, and Ca seen at the end of 21 d challenge in the matrix (Fig. 1B). Small negative influences of low pH exposure persisted in recovery for most ions, a trend particularly prominent in cells at Al = 0 μg/L. Small positive effects of water Ca and a slight parabolic influence of Al on whole body K and Ca also persisted.

Table 1 compares selected whole body ion levels after 21 d exposure and after 20 d recovery for three representative exposure combinations at Ca = 2 mg/L: control (pH = 6.5, Al = 0 μg/L), a cell (pH = 5.2, Al = 111 μg/L) where the stimulatory effect of Al had been greatest, and a cell (pH = 4.4, Al = 0 μg/L) where the depressant effect of acidity had been greatest. During the 20 d period of "recovery", whole body Na, Cl, K, and Ca levels all increased significantly in the control group as part of normal development (cf. figure 1 of Wood et al. 1990). In the group which had been exposed to severe acidity alone, electrolyte levels also increased substantially, but did not "catch up" to the levels of the controls. In contrast, the group which had been exposed to Al showed virtually no change during 20 d of recovery. Their electrolyte levels at the end of 21 d exposure were already at values attained 20 d later in the controls, and remained generally static over this period, except for Ca which increased slightly.

Swim-Up Fry — Relative Influence of pH, Ca, and Al

Figure 5 summarizes the results of the multiple regression analyses for the effects of pH, Ca, and Al on the various measured parameters in swim-up fry. Apart from the influence of water Al on whole body Na and ΔAl levels after 20 d recovery (Fig. 5B), all factors significant by multiple regression were also significant by factorial analysis of variance. At the end of 21 d exposure (Fig. 5A), overall patterns in swim-up fry were similar to those seen at this point with yolk-sac fry (cf. Fig. 1A), although the relative influence of water pH on whole body Na, Cl, and K was even greater, while that of water Al was reduced. In addition, the three experimental variables exerted a much greater effect on body weight and Mg content than seen with yolk-sac fry. After 20 d recovery (Fig. 5B), influences on whole body Na, Cl, and Ca levels persisted much more strongly than in yolk-sac fry (cf. Fig. 1B), a phenomenon which was most marked for the effects of both water Ca and pH on whole body Ca content.

Swim-Up Fry — Mortality and Body Weights

In terms of survival, swim-up fry were more sensitive than yolk-sac fry to low pH and Al (cf. Ingersoll et al. 1990a). Mortality was complete in all cells at pH = 4.0, in all cells at pH = 4.4 with Al = 1000 μg/L, and at pH = 5.2 with Al = 333 μg/L, Ca = 0.5 mg/L. Survival was also depressed from normal levels of 80–100% to 20–70% in some of the other more acidic cells, especially at lower Ca and higher Al levels. A further difference from the yolk-sac pattern was seen in recovery, for mortality continued during the recovery period at equal or higher levels than during exposure itself. Survival during the recovery period was strongly positively correlated with the water Ca of the exposure period.

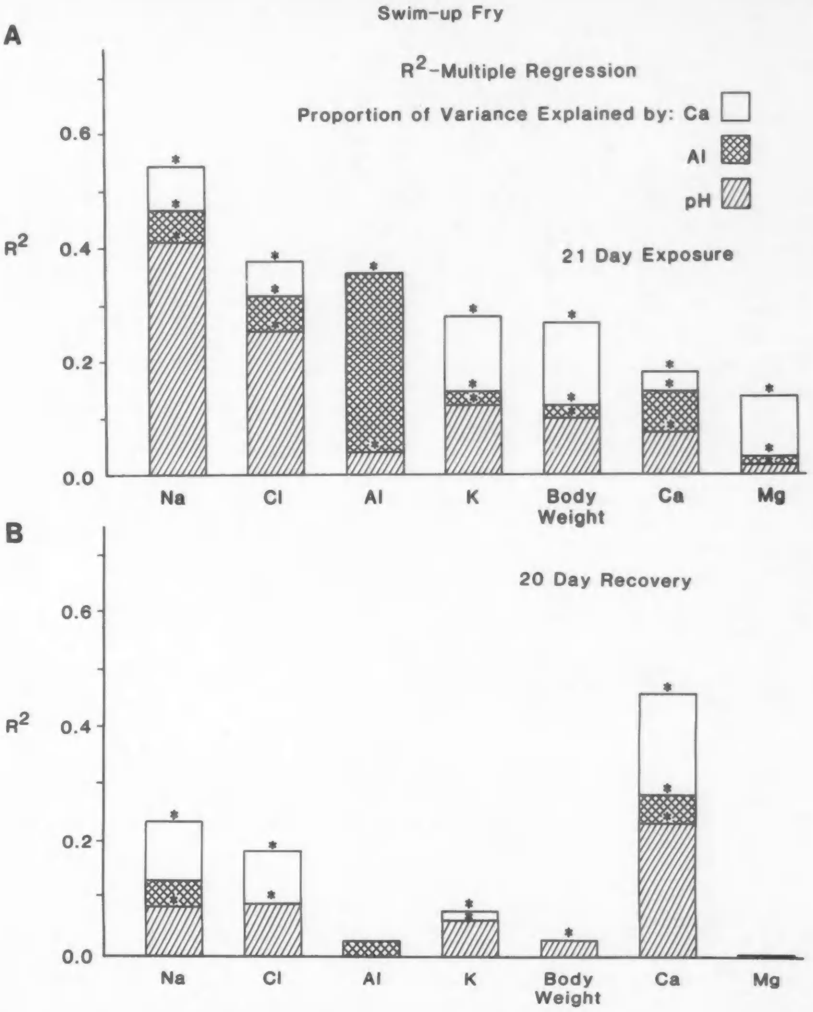

FIG. 5. Total variance explained (R^2) and proportion attributable to each factor (pH, Ca, Al of exposure matrix) as determined by stepwise multiple regression analysis, for various whole body parameters in swim-up fry of brook trout. Results are shown for fry at the end of 21 d exposure and following 20 d recovery under control conditions (pH = 6.5, Ca = 2 mg/L, Al = 0 μg/L). Asterisks indicate relationships which were also significant by factorial analysis of variance.

Again in contrast to yolk-sac fry, wet body weight of swim-up fry was strongly influenced by the exposure conditions, which accounted for almost 30% of the total variance (Fig. 5A). Wet weight was significantly decreased by low pH and raised by Ca, while Al exerted a weak parabolic effect in raising weight at intermediate levels. Dry weight was unaffected by Ca or Al, but was influenced in a biphasic fashion by pH, being slightly higher at pH = 4.4 and 6.5 than at 4.8 and 5.2. These weight trends were similar to those seen during the 91 d exposures (Wood et al. 1990; Ingersoll et al. 1990a). During recovery, the only significant weight effect was the persistence of a small negative influence of acidity on wet weight (Fig. 5B).

Swim-Up Fry — Whole Body Electrolytes after Challenge

The pH/Ca/Al exposures exerted major sublethal effects on whole body Na and Cl levels in swim-up fry; only the Na data are shown (Fig. 6), for Cl exhibited similar trends, though with greater variability. In the absence of Al, low pH alone lowered these extracellular electrolytes by as much as 60% in a linear fashion. In the matrix as a whole, pH was the major determinant of Na and Cl concentrations, accounting for 26% (Cl) to 41% (Na) of the variance (Fig. 5A). Calcium exerted a definite protective effect (proportional to log Ca), though this occurred only at acidic pH, and was indefinite at Al levels ≥37 μg/L (Fig. 6). Aluminum itself showed a weak tendency (parabolic with log

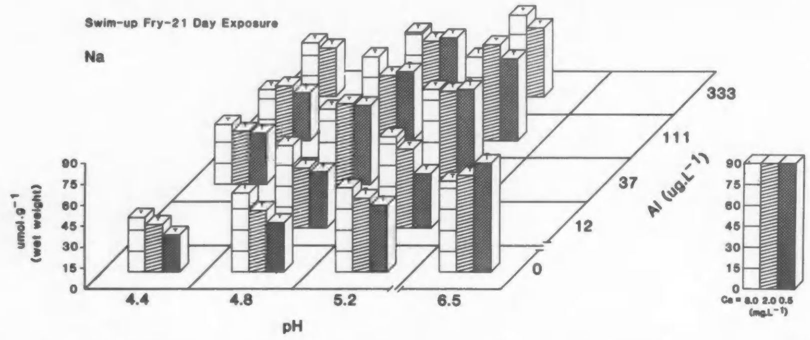

FIG. 6. Whole body concentrations of Na in swim-up fry of brook trout after 21 d exposure to a matrix of pH, Ca, and Al. Other details as in legend of Fig. 2.

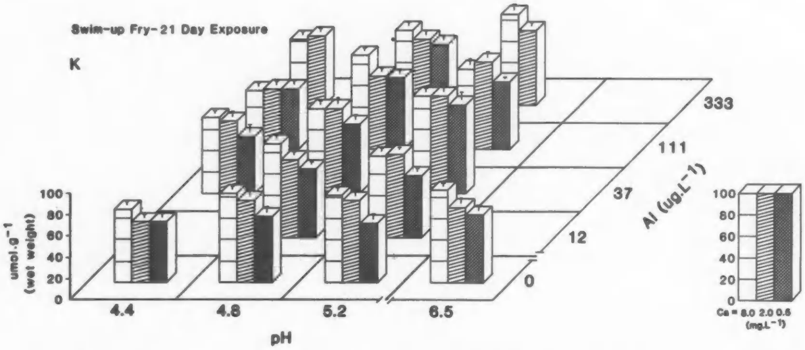

FIG. 7. Whole body concentrations of K in swim-up fry of brook trout after 21 d exposure to a matrix of pH, Ca, and Al. Other details as in legend of Fig. 2.

Al) to raise Na and Cl. While these trends were similar to those for yolk-sac fry (Fig. 2), the relative contribution of pH was greater and that of Al reduced. Furthermore, the presence of intermediate Al levels did not raise Na and Cl above the control values (Fig. 6).

The concentration of K, the major intracellular electrolyte, was also strongly affected by the 21 d exposure (Fig. 7). Potassium was lowered by acidity and raised by log Ca, the two factors contributing almost equally to the 30% of variance explained (Fig. 5A). Aluminum had only a very small parabolic effect, and did not elevate K above control values. Apart from the reduced influence of Al, these trends were similar to those seen in yolk-sac fry (Fig. 3).

Whole body levels of Ca (Fig. 8) were less affected by the 21 d exposure than those of Na, Cl, and K, although the effects of all three variables (pH, Ca, and Al) were still significant (Fig. 5A). These effects were also less than those seen in yolk-sac fry (Fig. 4). Whole body Ca concentrations were depressed by low pH, affected in a parabolic manner by Al, and very slightly raised by increasing Ca (Fig. 8).

Magnesium responded in an opposite fashion, being elevated by factors which had a deleterious effect on the other ions (Table 2). This was seen most clearly in the negative influence of log Ca, as well as in small negative effects of increasing pH and Al (Fig. 5A).

TABLE 2. A comparison of whole body Mg levels (μmol/g wet weight) in selected cells (Al = 0 μg/L) of the swim-up fry challenge experiment at the end of 21 d exposure. Means ± 1 SEM (*N*). * = $p \leq 0.05$ relative to comparable value at Ca = 2 mg/L; † = $p \leq 0.05$ relative to comparable value at pH = 4.4.

pH	Ca = 2 mg/L	Ca = 8 mg/L
4.4	16.6 ± 0.9 (10)	$13.0 \pm 1.0^{*}$ (9)
6.5	14.3 ± 1.6 (10)	$10.5 \pm 0.6^{*,\dagger}$ (10)

Swim-Up Fry — Whole Body Electrolytes after Recovery

The effects of 21 d exposure in the matrix persisted to a much greater extent in swim-up fry (Fig. 5B) than in yolk-sac fry (Fig. 1B). Indeed, for whole body Ca, the effects of pH and water Ca were far greater after recovery than at the end of the exposure itself (compare data in Fig. 8 and 9), despite the fact that the fish had been returned to constant control conditions for almost 3 wk. This comparison emphasizes the much higher absolute Ca concentrations at the end of 20 d recovery, reflecting the fact that the period from 91–111 d post-fertilization was a time of rapid Ca accumulation (cf. figure 1 of Wood et al. 1990). The delayed effects of water Ca on whole body Na and

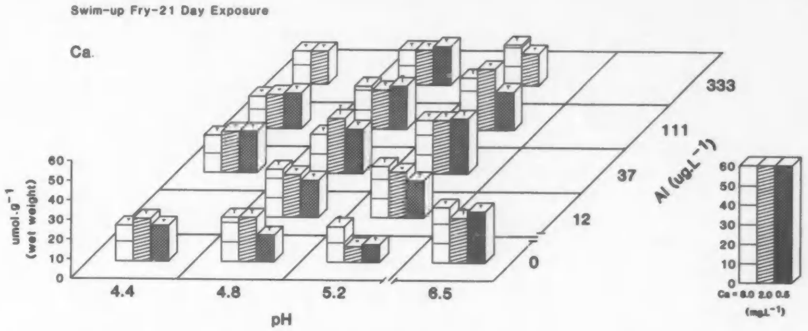

FIG. 8. Whole body concentrations of Ca in swim-up fry of brook trout after 21 d exposure to a matrix of pH, Ca, and Al. Other details as in legend of Fig. 2.

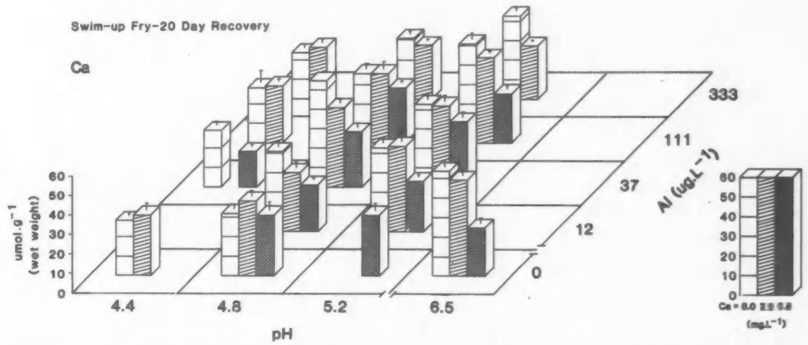

FIG. 9. Whole body concentrations of Ca in brook trout fry after 20 d recovery under control conditions (pH = 6.5, Ca = 2 mg/L, Al = 0 μg/L) following 21 d exposure in the swim-up stage to a matrix of pH, Ca, and Al. Means ± 1 SEM for 8–10 fish in most cells, falling as low as three in cells with high mortality.

TABLE 3. A comparison of whole body ion levels (μmol/g wet weight) in selected cells (Ca = 2 mg/L) of the swim-up fry challenge experiment at the end of 21 d exposure, and after 20 d of recovery under control conditions (pH = 6.5, Ca = 2 mg/L, Al = 0 μg/L). Means ± 1 SEM (*N*). * = $p \leqslant 0.05$ relative to comparable control value (pH = 6.5, Al = 0 μg/L) at this time; † = $p \leqslant 0.05$ relative to comparable value for this treatment at end of 21 d exposure.

		21 d exposure (day 70)			20 d recovery (day 90)		
pH	Al	Na	K	Ca	Na	K	Ca
6.5	0	69.2 ±2.8 (10)	70.1 ±4.3 (10)	22.7 ±2.5 (10)	$80.8^{\dagger}$ ±4.4 (10)	72.8 ±2.7 (10)	$48.8^{\dagger}$ ±2.9 (10)
5.2	37	66.4 ±2.4 (10)	69.5 ±7.7 (10)	26.6 ±1.2 (10)	$74.3^{\dagger}$ ±2.9 (9)	74.6 ±2.4 (9)	$41.8^{\dagger}$ ±3.0 (9)
4.4	0	33.7^{*} ±1.5 (10)	56.9^{*} ±3.7 (10)	21.6 ±1.6 (10)	$50.3^{\dagger,*}$ ±3.3 (5)	62.2 ±7.4 (5)	$31.0^{\dagger,*}$ ±3.5 (5)

Cl were also greater than those seen at the end of the challenge (Fig. 5B). The inhibitory effects of acidity on Na, Cl, and K, while attenuated, remained highly significant at the end of recovery (Fig. 5B). As in yolk-sac fry, this reflected a failure of the acid-exposed fish to "catch-up" to the controls during the recovery period (Table 3). There were no persistent effects on Mg (Fig. 5B), which continued to decline during the recovery period, in contrast to the other ions.

Aluminum Accumulation and Loss

Significant ΔAl was detected only at water Al ≥ 111 μg/L in both yolk-sac and swim-up fry after 21 d exposure (Fig. 10). Over the matrix as a whole, water Al was the major determinant of accumulation (Fig. 1A, 5A), but in cells at Al ≥ 111 μg/L, both Ca and acidity had strong negative influences on ΔAl. These trends and the values of ΔAl in comparable cells were similar in the two stages (Fig. 10), as well as in the alevins exposed for 91 d (cf. figure 10 of Wood et al. 1990). During 20 d recovery in Al-free water at pH = 6.5, the accumulated load was lost almost completely, suggesting surficial adsorption onto, rather than into the fish. In the swim-up group, approximately 5% of the original ΔAl persisted after 20 d recovery in fish from several exposure combinations, giving rise to the slight positive correlation shown in Fig. 5B. However, the biological significance is questionable; these values approximated the detection limit of our analytical technique, and the effect was not significant by factorial analysis of variance (Fig. 5B).

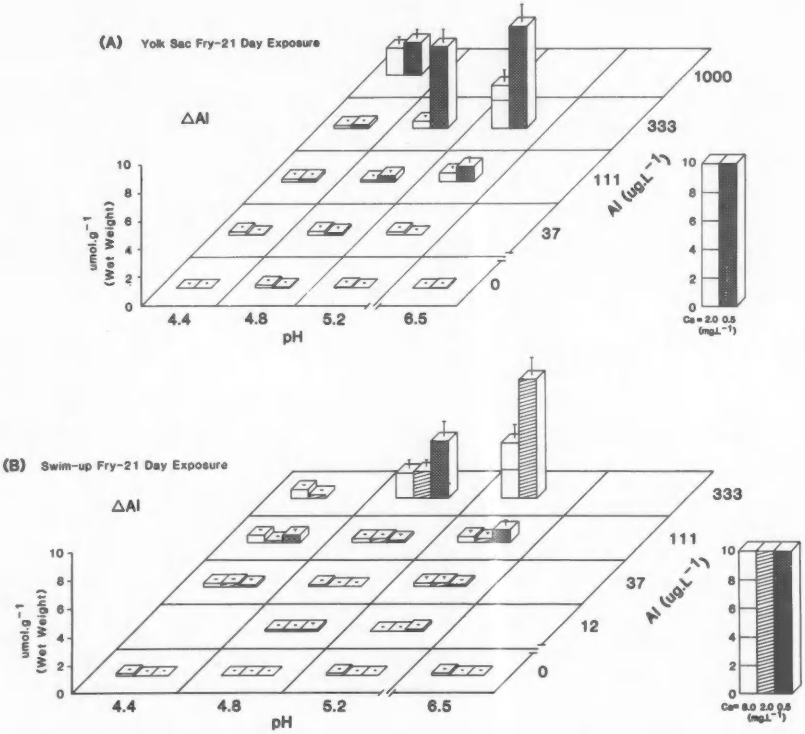

FIG. 10. Whole body accumulation of aluminum (ΔAl) in (A) yolk-sac fry and (B) swim-up fry of brook trout after 21 d exposure to a matrix of pH, Ca, and Al. Other details as in legend of Fig. 2.

Discussion

Relative Importance of pH and Ca in Different Exposure Regimes

In both yolk-sac and swim-up fry exposed for 21 d in the matrix, water pH was the principal determinant of whole body ion status for most electrolytes (Fig. 1A, 5A). This directly contrasts with the responses of alevins raised continuously from fertilization for 91 d in the matrix, where water Ca was the principal determinant of whole body ion status (cf. figure 2 of Wood et al. 1990). To some extent, these different conclusions are a function of the different experimental designs.

The first difference relates to the fact that our experiments measured only sublethal responses; fry exhibiting lethal responses were lost prior to sampling and did not contribute their presumably more severe ionic changes to the data pool. In other words, selection eliminated the most extreme responders from the data set.The cells where the sublethal pH effects were most extreme in the 21 d challenges (at pH = 4.4, and at pH = 4.8, low Ca; eg. Fig. 2, 3, 4, 6, 7, and 8) were lost entirely in the 91 d exposures and did not contribute data to the 91 d analysis (cf. figure 5, 6, 7, 8, and 9 of Wood et al. 1990). In other low pH cells where 91 d survivors were available for analysis, these survivors were presumably those fish most resistant to the effects of acidity. The second difference is that the range of water Ca concentrations examined in the present age-specific challenges was less extensive (two or three Ca levels) than in the 91 d exposures (four Ca levels), which could have reduced the influence of Ca in the factorial analysis. To test whether this was the case, the data from the 91 d exposures were re-analyzed using only those two or three Ca levels employed in the yolk-sac and swim-up experiments. The influence of Ca (in terms of R^2) was unaltered when three Ca levels were employed (0.5, 2, and 8 mg/L as in the swim-up exposures), but was generally reduced when only two Ca levels were employed (0.5 and 2 mg/L as in the yolk-sac exposures). Nevertheless, Ca remained the dominant factor for all five whole body ions.

These two differences notwithstanding, the results of the two sets of experiments remain fundamentally different from one another, and suggest that the responses in a field situation will depend critically on the nature of the exposure. Fry surviving a continuous 91 d exposure from fertilization will maintain an ionic status most dependent upon the water Ca during the exposure, while alevins surviving a 21 d challenge in the yolk-sac or swim-up phase will exhibit a status most dependent upon the pH of the challenge. As outlined in the Introduction and below, the latter situation (episodic exposure, especially of the swim-up stage) appears to be the more environmentally realistic.

Relative Importance of Al in Different Exposure Regimes

While never as pronounced as that of pH, the curious parabolic influence of Al was an important determinant of whole body ion status in these episodic exposures, especially in the yolk-sac stage (Fig. 1, 5). This influence was largely positive, and in many cells offered a greater protection against the deleterious effects of low pH than did higher levels of Ca. This protective and/or stimulatory action of Al in both the 21 d and 91 d exposures (Wood et al. 1990) was surprising, for toxic effects have usually been associated with the presence of Al at low pH (cf. Schofield and Trojnar 1980; Brown 1983; Gunn and Noakes 1987; McDonald et al. 1988). Positive effects were most pronounced with Al levels of 37 and 111 $\mu g/L$ at $pH=4.8$ and 5.2, especially in the yolk-sac fry. In these cells, whole body Na, Cl, K, and Ca concentrations were raised well above those in the control yolk-sac fry at $pH=6.5$, $Al=0$ $\mu g/L$ (Fig. 2, 3, 4). Negative effects were seen only rarely in surviving alevins, and only at the highest concentrations of Al (333, 1000 $\mu g/L$) where the metal was clearly accumulated by the fish (Fig. 10). It is worth noting that water Al levels measured in the exposure chambers were always in close agreement with nominal values in the present experiments (Ingersoll et al. 1990a; Wood et al. 1990).

The mechanism of action for the negative effects of Al is probably the same as in adult salmonids — a combination of inhibition of active ion influx through the "chloride cells" and stimulation of passive efflux through paracellular channels (cf. Booth et al. 1988; McDonald and Milligan, 1988). The influx effect may result from direct inhibition of transport enzymes by Al (Staurnes et al. 1984), and the efflux effect from an inflammation and edema of the transporting epithelia in response to surficial Al build-up (Karlsson–Norrgren et al. 1986a, b). Both of these effects would be additional to the separate actions of acidity alone in inhibiting active influx and stimulating passive efflux.

The explanation for the positive effects of Al is less clear. Several previous studies have demonstrated that Al may protect against the effects of severe acidity ($pH \leq 4.5$; Muniz and Leivestad 1980; Schofield and Trojnar 1980; Baker and Schofield 1982; Hunn et al. 1987), an action attributed to the ability of polyvalent cations such as Al^{3+} to stabilize membrane permeability in much the same manner as Ca^{2+}. However at $pH=4.8–5.2$, where effects were most prominent in the present study, total $Al=37–111$ $\mu g/L$ would yield free Al^{3+} concentrations of only about 0.3–2.1 $\mu mol/L$ (McDonald et al. 1989), relative to $Ca^{2+}=12.5–200$ $\mu mol/L$. It seems unlikely that such low levels of free Al^{3+} could be solely responsible for complete protection against the toxic effects of low pH on ionoregulation, let alone for stimulation of whole body ions above control levels.

Nevertheless, a similar protective influence of comparable levels of Al (75–150 $\mu g/L$) was seen in adult brook trout exposed to $pH=5.2$ for 70 d (Wood et al. 1988a, b). An alternative or additional explanation is the proliferation of "chloride cells" on the branchial epithelium of adult salmonids seen during comparable long-term acid/Al exposures (Karlsson–Norrgren et al. 1986a, b; Tietge et al. 1988), which is thought to explain the greater Na uptake capacity of such fish (McDonald and Milligan 1988). It is unknown whether similar "chloride cell" proliferation occurs on the transporting epithelia (vitelline membrane, developing gills) of alevins. Ingersoll et al. (1990b) could detect no significant effect of Al on mucous cells in the general body epithelia of brook trout alevins in exposures similar to those of the present study.

A final possibility is that Al in some way accelerated development, for increases in the concentrations of Na, Cl, K, and Ca occurred as part of normal ontogeny (cf. figure 1 of Wood et al. 1990). This explanation would account for whole body ions remaining almost static over the 20 d recovery period in yolk-sac fry previously exposed to stimulatory levels of Al (Table 1). However, other data in the present study suggest that developmental rates were unaffected in the yolk-sac stage where the greatest stimulatory effects of Al occurred (see below). Furthermore, in other investigations at comparable pH, Al clearly inhibited both development and mineralization of salmonid fry (Hunn et al. 1987; Gunn and Noakes, 1987). In summary, the mechanism(s) remain problematical; there is a clear need for further mechanistic studies on this unusual protective/stimulatory action of sublethal Al on ionoregulation in both alevins and adults.

Ionoregulatory Effects vs. Developmental Effects

As normal development proceeds in these early life stages, there are substantial increases in the whole body concentrations of most electrolytes, but decreases in body weight and Mg levels as the yolk is resorbed (Hayes et al. 1946; Rombough and Garside 1984; Geen et al. 1985; Peterson and Martin–Robichaud 1986; Gunn and Noakes 1987; Wood et al. 1990). In the continuous 91 d exposures (Wood et al. 1990), it was not possible to separate ionic responses caused by direct actions of the exposure conditions on ion exchange processes from those arising indirectly via changes in developmental rate. However, in the present 21 d challenges, there was evidence for the occurrence of both phenomena, in agreement with the findings of Peterson and Martin–Robichaud (1986) on *Salmo salar* alevins at low pH.

In the yolk-sac fry, the 21 d exposure had pronounced effects on whole body Na, Cl, K, and Ca concentrations, but negligible influence on either Mg concentrations or body weight (Fig. 1A). This suggests that developmental effects were insignificant and that changes in whole body ions resulted largely from direct actions of pH, Ca, and Al on transport and permeability processes in yolk-sac fry. However, in swim-up fry there were substantial effects of the matrix on both body weight and Mg levels (Table 2) in addition to those on the other whole body ions (Fig. 5A). Exposure conditions which tended to lower Na, Cl, K, and Ca (i.e. low pH, low Ca, high Al) simultaneously tended to raise Mg and lower wet body weight, while dry body weight varied in a biphasic fashion with pH. These patterns were very similar to those seen during the 91 d exposures (cf. Wood et al. 1990) where they reflected delayed yolk-resorption, reduced feeding, inhibited growth, and increased metabolic costs under adverse conditions. Thus the exposure conditions clearly exerted developmental actions on the swim-up stages which may have made some contribution to the overall ionic effects. Furthermore, these findings suggest that at least some of the developmental effects seen at the end of 91 d continuous exposure occurred during the swim-up phase.

Lacroix et al. (1985) followed growth and whole body ion levels in larval *Salmo salar* exposed in situ to natural acidic conditions. In terms of developmental stage, their exposures started at about the point where the present experiments terminated, so their fish undoubtedly relied more on exogenous feeding than the present fry. In agreement with the present findings, exposure to $pH \simeq 5.0$ in soft water ($Ca \simeq 0.6$ mg/L) with high Al ($\simeq 200$ $\mu g/L$) induced depressions in most whole body ions and produced a retardation of development. Feeding and growth were depressed, and eventual emaciation resulted. As the ionic effects tended to precede the developmental effects, there was evidence for at least a partial separation of the two phenomena.

Recovery

Only a few studies have examined the physiology of recovery from acid stress for more than several days in juvenile or adult fish (Leivestad et al. 1976; Brown et al. 1986; Jones et al. 1987). In general, these indicated complete recovery within 7–14 days. Only one investigation has examined recovery in early life stages after acid or acid plus Al exposure. Gunn and Noakes (1987) reported significant negative effects on whole body ions, development, and skeletal calcification in lake trout alevins (*S. namaycush*) after 21–32 d recovery from a 5 d pulse exposure to Al = 100 or 200 μg/L at pH = 5.0 in the yolk-sac stage. There was no persistent effect of exposure to this single low pH (5.0) alone; the influence of water Ca was not examined. In the present study on brook trout alevins (*S. fontinalis*), deleterious effects persisted after 20 d of recovery from 21 d pulse exposures to low pH and low Ca conditions (Tables 1, 3; Fig. 1B, 5B). This persistence was especially prominent in swim-up fry, where both mortality and negative effects on whole body Ca were greater during recovery than during the original exposure (Fig. 5B, 8, 9). Interestingly, the protective effects of Al, which were directly opposite those reported by Gunn and Noakes (1987) in lake trout fry, also exhibited some persistence in recovering brook trout swim-up fry, though the marked stimulatory effects of Al largely disappeared in recovering brook trout yolk-sac fry (Table 1).

These differences from previous findings on older fish undoubtedly reflect the fact that exposure during the yolk-sac and swim-up stages coincided with a normal period of intense accumulation of ions. When this accumulation was blocked by either low pH or low Ca, the surviving fry failed to "catch-up" to controls which had accomplished a substantial net uptake over this same period. The effect was particularly pronounced when the adverse conditions of the exposure also delayed development, as during the swim-up stage. The remarkable effects on whole body Ca during the swim-up recovery (Fig. 9) presumably resulted from a combination of inhibited uptake during exposure (Fig. 8), inhibited development, and coincidence of the recovery period with a normal period of high Ca uptake (cf. figure 1 of Wood et al. 1990).

Relative Sensitivity of Yolk-Sac vs. Swim-Up Fry

In terms of relative effects on whole body ions after 21 d exposure to the matrix, the responses of yolk-sac and swim-up fry were quantitatively similar, apart from the more pronounced protective/stimulatory influence of Al in the former. This suggests that the toxic actions of H^+ and the protective actions of Ca are similar on vitelline and on branchial membranes, while the balance between protection and toxicity by Al is shifted slightly more towards the latter in the branchial epithelium. However, in terms of survival, body weight, development, and the persistence of deleterious ionoregulatory effects during recovery, the swim-up stage was clearly the more sensitive. The explanation probably relates to the increased importance of the gills in the swim-up stage for both ion uptake and respiratory gas exchange.

Associated with this transition from vitelline to branchial membranes as the principal exchange site will be an increasing reliance on the external environment and a decreasing reliance on yolk-sac stores for both minerals and nutrients to fuel further development. Therefore it is not surprising that the effects of toxicants in this external environment became more severe as development progressed. Other studies have reported sensitivity patterns both in agreement and disagreement with those of the present study (reviewed by Ingersoll et al. 1990a). Such discrepancies probably result from differences in temperature which will affect developmental rates, and in the balance of Al relative to H^+ as the primary toxic agent. Based on mortality data, Ingersoll et al. (1990a) detected a general trend for Al toxicity to increase and H^+ toxicity to decrease as development proceeded in the present experiments.

Environmental Significance

As outlined in the Introduction and discussed in detail by Trojnar (1977) and Gunn (1986), the yolk-sac and swim-up stages tested in the present experiments are probably the critical life stages for brook trout in terms of susceptibility to episodic acid pulses. For example, in acid-impacted Ontario streams, fry emerging from the relatively sheltered conditions of the redd may experience sudden pH decreases of 0.5–2.0 units, increases in total Al from background levels to over 150 μg/L, and substantial decreases in Ca (Keller 1983; Gunn 1986). The present experiments are therefore representative of such conditions.

The most important conclusion of these experiments is that water pH will be the major factor affecting whole body ion levels in fry emerging into an acidic environment. For example, if this emergence coincided with the swim-up stage of the present experiments,and the ambient water was at pH = 4.8 compared to an interstitial pH = 6.5 in the redd (unchanged Ca = 2 mg/L, Al = 0 μg/L), then whole body Na and Cl would be depressed by 25–35% in survivors, and K and Ca by about 5% relative to fry maintained in the interstitial conditions. After 20 d recovery at pH = 6.5, the Na, Cl, and K depressions would persist at the same relative magnitude, though they would be absolutely larger due to the general increase in whole body ion levels over this period. At the same time, the depression in whole body Ca would reach 25%. In the unlikely event the ambient water was at Ca = 8 mg/L during this acidic pulse, these effects would be slightly attenuated. However, if, as is more probable, the acidic water was at a lower Ca (e.g. 0.5 mg/L) than in the redd, then the Na, Cl, and K depressions would be slightly exacerbated, while the Ca depression would reach almost 40%. Thus, while water Ca has a significant influence, it is certainly less important than pH in episodic exposures, in contrast to the situation of continuous exposure from fertilization through swim-up (Wood et al. 1990). Beggs and Gunn (1986) found pH alone to be a good indicator of the presence/absence (threshold pH = 5.0) of brook trout populations in Ontario lakes.

A surprising finding of these experiments is the largely positive, rather than negative, influence of Al at low pH on whole body ions in brook trout alevins. This contrasts with the recent findings of Gunn and Noakes (1987) on lake trout fry; the only obvious reasons for this discrepancy are differences in fish species and exposure regime. In the scenario outlined above, whole body Na and Cl levels of brook trout fry would be depressed by only 15–20% after the recovery period if the acidic water (pH = 4.8, Ca = 2 mg/L) into which the fry emerged also contained Al = 111 μg/L. As yet there appears to be no evidence that Al can actually protect in this manner in the field situation. There are also no field data to confirm two other interesting laboratory observations which complicate extrapolation of the present data to the field. Firstly, Gunn and Noakes (1986) have shown that swim-up fry of *Salvelinus fontinalis* can actively avoid water of low pH and high Al content. Secondly, the mortality data of Trojnar (1977) indicate that fry pre-exposed to acidic conditions in the redd may be more tolerant of severe acidity during emergence. There is a clear need for more field trials at brook trout spawning sites, of the type pioneered by Gunn and Keller (1984a, b) with *Salvelinus namaycush* redds and Lacroix (1985) with *Salmo salar* redds, especially in combination with physiological and behavioral measurements (e.g. Lacroix et al. 1985).

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References

Baker, J. P., and C. L. Schofield. 1982. Aluminum toxicity to fish in acidic waters. Water Air Soil Pollut. 18: 289–310.

Beggs, G. L., and J. M. Gunn. 1986. Response of lake trout (*Salvelinus namaycush*) and brook trout (*S. fontinalis*) to surface water acidification in Ontario. Water Air Soil Pollut. 30: 711–717.

Booth, C. E., D. G. McDonald, B. P. Simons, and C. M. Wood. 1988. Effects of aluminum and low pH on net ion fluxes and ion balance in the brook trout, *Salvelinus fontinalis*. Can. J. Fish. Aquat. Sci. 45: 1563–1574.

Brown, D. J. A. 1983. Effect of calcium and aluminium concentrations on the survival of brown trout (*Salmo trutta*) at low pH. Bull. Environ. Contam. Toxicol. 30: 582–587.

Brown, S. B., R. E. Evans, and T. J. Hara. 1986. Interrenal, thyroidal, carbohydrate, and electrolyte responses in rainbow trout (*Salmo gairdneri*) during recovery from effects of acidification. Can. J. Fish. Aquat. Sci. 43: 714–718.

Christophersen, N., S. Rustad, and H. M. Seip. 1984. Modelling streamwater chemistry with snowmelt. Philos. Trans. R. Soc. Lond. B305: 427–439.

Geen, G. H., J. D. Neilsen, and M. Bradford. 1985. Effects of pH on the early development and growth and otolith microstructure of chinook salmon, *Oncorhynchus tshawytscha*. Can. J. Zool. 63: 22–27.

Gunn, J. M. 1986. Behaviour and ecology of salmonid fishes exposed to episodic pH depressions. Environ. Biol. Fish. 17: 241–252.

Gunn, J. M., and W. Keller. 1984a. Spawning site water chemistry and lake trout (*Salvelinus namaycush*) sac fry survival during spring snowmelt. Can. J. Fish. Aquat. Sci. 41: 319–329.

1984b. *In situ* manipulation of water chemistry using crushed limestone and observed effects on fish. Fisheries 9: 19–24.

Gunn, J. M., and D. G. Noakes. 1986. Avoidance of low pH and elevated Al concentrations by brook charr (*Salvelinus fontinalis*) alevins in laboratory tests. Water Air Soil Pollut. 30: 497–503.

1987. Latent effects of pulse exposure to aluminum and low pH on size, ionic composition, and feeding efficiency of lake trout (*Salvelinus namaycush*) alevins. Can. J. Fish. Aquat. Sci. 44: 1418–1424.

Hayes, F. R., D. A. Darcy, and C. M. Sullivan. 1946. Changes in the inorganic constituents of developing salmon eggs. J. Biol. Chem. 163: 621–633.

Hunn, J. B., C. L. Cleveland, and E. E. Little. 1987. Influence of pH and aluminum on developing brook trout in low calcium water. Environ. Pollut. 43: 63–73.

Ingersoll, C. G., D. Gulley, D. R. Mount, T. W. La Point, and H. L. Bergman. 1990a. Effects of pH, aluminum, and calcium on survival and growth of eggs and fry of brook trout (*Salvelinus fontinalis*). 47: 1580–1592.

Ingersoll, C. G., D. A. Sanchez, J. S. Meyer, D. Gulley, and J. E. Tietge. 1990b. Epidermal response to pH, aluminum, and calcium exposure in brook trout (*Salvelinus fontinalis*) fry. 47: 1616–1622.

Johnson, D. W., and D. A. Webster. 1977. Avoidance of low pH in selection of spawning sites by brook trout (*Salvelinus fontinalis*). J. Fish. Res. Board Can. 34: 2215–2218.

Jones, K. A., S. B. Brown, and T. J. Hara. 1987. Behavioural and biochemical studies of onset and recovery from acid stress in arctic char (*Salvelinus alpinus*). Can. J. Fish. Aquat. Sci. 44: 373–381.

Karlsson–Norrgren, L., W. Dickson, O. Ljungberg, and P. Runn. 1986a. Acid water and aluminium exposure: gill lesions and aluminum accumulation in farmed brown trout, *Salmo trutta* L. J. Fish. Dis. 9: 1–10.

Karlsson–Norrgren, L., I. Bjorklund, O. Ljungberg, and P. Runn. 1986b. Acid water and aluminium exposure: experimentally induced gill lesions in brown trout, *Salmo trutta* L. J. Fish. Dis. 9: 11–25.

Keller, W. 1983. Spring pH and alkalinity depressions in Lake Superior tributaries. J. Great Lakes Res. 9: 425–429.

Lacroix, G. L. 1985. Survival of eggs and alevins of Atlantic salmon (*Salmo salar*) in relation to the chemistry of interstitial water in redds in some acidic streams of Atlantic Canada. Can. J. Fish. Aquat. Sci. 42: 292–299.

Lacroix, G. L., D. J. Gordon, and D. J. Johnston. 1985. Effects of low environmental pH on the survival, growth, and ionic composition of postemergent Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 42: 768–775.

Leivestad, H., G. Hendrey, I. P. Muniz, and E. Snekvik. 1976. Effects of acid precipitation on freshwater organisms, p. 87–111. *In* F. H. Braekke [ed.] Impact of acid precipitation on forest and freshwater ecosystems in Norway. SNSF Project FR6/76.

Marmorek, D. R., G. Cunningham, M. L. Jones, and P. Bunnell. 1985. Snowmelt effects related to acidic precipitation: a structured review of existing knowledge and current research activities. LRATP Workshop. Atmospheric Environment Service, Downsview, Ont.

McDonald, D. G., and C. L. Milligan. 1988. Sodium transport in the brook trout, *Salvelinus fontinalis*: effects of prolonged exposure to low pH in the presence and absence of aluminum. Can. J. Fish. Aquat. Sci. 45: 1606–1613.

McDonald, D. G., J. P. Reader, and T. K. R. Dalziel. 1989. The combined effects of pH and trace metals on fish ionoregulation, p. 221–242. *In* R. Morris, D. J. A. Brown, E. W. Taylor, and J. A. Brown [ed.] Acid Toxicity and Aquatic Animals, Society for Experimental Biology Seminar Series. Cambridge University Press, UK.

Muniz, I. P., and H. Leivestad. 1980. Toxic effects of aluminum on the brown trout, *Salmo trutta* L. p. 320–321. *In* D. Drablos and A. Tollan [ed.] Ecological impact of acid precipitation. SNSF-Project.

Peterson, R. H., and D. J. Martin-Robichaud. 1986. Growth and major inorganic cation budgets of Atlantic salmon alevins at three ambient acidities. Trans. Am. Fish. Soc. 115: 220–226.

Rombough, P. J., and E. T. Garside. 1984. Disturbed ion balance in alevins of Atlantic salmon *Salmo salar* chronically exposed to sublethal concentrations of cadmium. Can. J. Zool. 62: 1443–1450.

Schofield, C. L., and R. J. Trojnar. 1980. Aluminum toxicity to brook trout (*Salvelinus fontinalis*) in acidified waters, p. 341–366. *In* T. Y. Toribara, M. W. Miller, and P. E. Morrow [ed.] "Polluted Rain" Plenum Press, New York, NY.

Staurnes, M., T. Sigholt, and O. B. Reite. 1984. Reduced carbonic anhydrase and Na^+ K^+-ATPase activity in gills of salmonids exposed to aluminum-containing acid water. Experimentia 40: 226–227.

Tietge, J. E., R. D. Johnson, and H. L. Bergman. 1988. Morphometric changes in gill secondary lamellae of brook trout (*Salvelinus fontinalis*) after long-term exposure to acid and aluminum. Can. J. Fish. Aquat. Sci. 45: 1643–1648.

Trojnar, J. R. 1977. Egg hatchability and tolerance of brook trout (*Salvelinus fontinalis*) fry at low pH. J. Fish. Res. Board Can. 34: 574–579.

Webster, D. A., and G. Eiriksdottir. 1976. Upwelling water as a factor influencing choice of spawning sites by brook trout, *Salvelinus fontinalis*. Trans. Am. Fish. Soc. 91: 168–174.

Wood, C. M., D. G. McDonald, C. E. Booth, B. P. Simons, C. G. Ingersoll, and H. L. Bergman. 1988a. Physiological evidence of acclimation to acid/aluminum stress in adult brook trout (*Salvelinus fontinalis*). 1. Blood composition and net sodium fluxes. Can. J. Fish. Aquat. Sci. 45: 1587–1596.

Wood, C. M., B. P. Simons, D. R. Mount, and H. L. Bergman. 1988b. Physiological evidence of acclimation to acid/aluminum stress in adult brook trout (*Salvelinus fontinalis*). 2. Blood parameters by cannulation. Can. J. Fish. Aquat. Sci. 45: 1597–1605.

Wood, C. M., D. G. McDonald, C. G. Ingersoll, D. R. Mount, O. E. Johannsson, S. Landsburger, and H. L. Bergman. 1990. Effects of water acidity, calcium and aluminum on whole body ions of brook trout (*Salvelinus fontinalis*) continuously exposed from fertilization to swim-up: a study by instrumental neutron activation analysis. 47: 1593–1603.

Epidermal Response to pH, Aluminum, and Calcium Exposure in Brook Trout (*Salvelinus fontinalis*) Fry

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Eyed brook trout eggs were exposed to various combinations of pH (4.3–6.3), aluminum (0–1000 μg/L), and calcium (0.5 to 8.0 mg/L) for 40 d through hatching until the swim-up life stage. High resolution light microscopy, image analysis, and stereological techniques were used to quantitatively determine morphological changes in the epidermis of fry surviving this exposure. Exposure to increased acidity resulted in both mucous cell hypertrophy (increase in size) and hyperplasia (increase in number); exposure to low calcium resulted in mucous cell hyperplasia. Aluminum did not significantly affect mucous cell size or number. Epidermal thickness was not consistently affected by exposure to pH, aluminum, or calcium. The changes observed in epidermal mucous cells may be a compensatory mechanism used by brook trout fry to counter ionoregulatory stress resulting from extended exposure to acidic conditions.

Des oeufs embryonnés d'omble de fontaine ont été exposés à différentes combinaisons de pH (4,3 à 6,3), de concentrations d'aluminium (0 à 1000 μg/l) et de calcium (0,5 à 8,0 mg/L) pendant 40 jours, depuis l'éclosion jusqu'au stade d'alevin. La microscopie optique à haute résolution, l'analyse d'image et des techniques stéréologiques ont été utilisées pour mesurer les changements morphologiques de l'épiderme des alevins qui survivaient à cette exposition. Une exposition à une acidité accrue se traduisait par une hypertrophie des cellules muqueuses (augmentation de la taille) et une hyperplasie (augmentation du nombre); une exposition à une faible teneur en calcium provoquait une hyperplasie des cellules muqueuses. L'aluminium ne modifiait pas de façon significative la taille ou le nombre des cellules muqueuses. Une exposition à une variation du pH, à l'aluminium ou au calcium ne modifiait pas de façon constante l'épaisseur de l'épiderme. Les modifications observées au niveau des cellules muqueuses de l'épiderme peuvent être un mécanisme de compensation de l'alevin d'omble de fontaine pour compenser l'agression ionorégulatrice à la suite d'une exposition prolongée à des conditions d'acidité.

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Increased acidity of lakes and streams in Scandinavia (Wright and Snekvik 1978), eastern Canada (Kelso and Minns 1982), and the northeastern United States (Schofield 1982) has led to the decline of fish populations in some of these areas (Baker 1984). The mortality of early life stages of fish, especially newly emerged fry, may be the cause of these declines (Muniz and Leivestad 1980; Gunn and Keller 1984).

The integument of larval fish is a major site for ion exchange and respiration (Peterson and Martin–Robichaud 1986). Damage to this tissue resulting from acidic conditions may be one of the causes of death during early life stages of fish (Daye and Garside 1980). Only as the gills of older fish develop secondary lamellae, may the disruption of branchial epithelium become a prime cause of death with exposure to acidification (Rombough 1982).

Increased mucus production by mucous cells may be a compensatory mechanism used by fish to counter ionoregulatory stress (Van Oosten 1957; Harris and Hunt 1973; Agarwal et al. 1979; Van de Winkle et al. 1986). Fish exposed to acid or aluminum in the laboratory may experience either epidermal mucous cell hypertrophy or hyperplasia (Daye and Garside 1976, 1980; Zuchelkowski et al. 1981, 1986; Tandjung 1982; Segner et al. 1979, 1988). However, the findings of these previous studies are difficult to relate to effects on fish populations in acid-sensitive waters because the exposures either: (1) used hard water or extremely elevated aluminum concentrations not representative of acidified natural waters, (2) tested species of fish which do not normally inhabit acidified natural waters,(3) started with older life stages of fish which were less sensitive to the effects of acidification, (4) did not address the interactive effects of pH, aluminum and calcium, or (5) were short-term acute exposures.

The objectives of the present study were to: (1) investigate the epidermal responses of brook trout (*Salvelinus fontinalis*) fry after extended exposure to combinations of pH, aluminum, and calcium levels (Ingersoll et al. 1990) representative of acid-sensitive natural waters (e.g. Schofield 1982) and (2) develop high resolution light microscopy, image analysis, and stereological techniques (Weibel 1979) to quantitatively determine these histopathological changes. Additional papers in this issue and in a previous issue (Bergman et al. 1988) present toxicology, physiology, and histopathology results of studies with early life stage, juvenile, and adult brook trout exposed in the laboratory to water conditions typical of acidified lakes and streams.

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Materials and Methods

Exposure

Eyed eggs of brook trout were exposed to 64 combinations of pH (as H_2SO_4: 4.3–6.3), aluminum (as $AlCl_3$: 0–1000 μg/L), and calcium (as $CaCl_2$: 0.5–8.0 mg/L) for 40 d through hatching, until the swim-up stage (see Ingersoll et al. 1985, 1990; and Ingersoll 1986, for a detailed description of the exposure). At the end of this exposure fry were sampled from 14 of these 64 combinations for histopathological evaluation (Table 1).

Fixation, Embedment, and Sampling

Whole fry were fixed for 24 h in a 2.5% buffered glutaraldehyde solution and post-fixed for 3.5 h in a 1% buffered-solution (buffer: 0.075 M HEPES at pH 7.4, 4°C). After dehydration in a series of ethanol rinses (50, 75, 95 and 100%) and propylene oxide (100%), fish were embedded individually in Araldite-502 epoxy resin which was polymerized at 60°C for 24 h. One-micrometer-thick cross sections of oriented whole fish were taken anterior to the dorsal fin with an ultramicrotome and stained for 1 min with 0.2% azure-B (0.25 M sodium borate at pH 8.1). Measurements of stained sections were made using Nomarski differential interference contrast at a total magnification of 2632×. Epidermal structures were quantified using a video camera, video monitor, Bioquant II image analysis system (R&M Biometrics, Nashville, TN), and a digitizing tablet (Houston Instrument Model DT11, Austin, TX).

Four measurements were made along the epidermal cross section between the left and right lateral line canals of each fish: (1) mucous cell volume density, (2) mucous cell diameter, (3) number of mucous cells/mm of epidermis, and (4) epidermal thickness. We were also interested in determining effects of epidermal chloride cells (Tietge et al. 1988); however, chloride cells were only occasionally observed in fish across all of the exposure treatments.

The volume density of mucous cells (percent of the epidermis consisting of mucous cells) was estimated by placing a grid with random points over the video monitor and counting: (1) the number of points intersecting mucous cells and (2) the number of points intersecting epidermis. Volume density was then calculated by the equation $Vv = Mp/Ep \times 100\%$, where Vv = epidermal mucous cell volume density (%), Mp = the number of points intersecting mucous cells, and Ep = the total number of points intersecting epidermis (DeHoff and Rhines 1968, Elias et al. 1971, Weibel 1979, Aherne and Dunnill 1982).

The diameter of mucous cells in profile ($n > 25$ cells/fish) was measured from the most exterior to the most interior margin of each cell in cross section. Mucous cell diameter was then calculated by the equation $D = (4/\pi)^*d$, where D = mean cell diameter, and d = mean cell profile diameter (Weibel 1979). The number of mucous cells/mm of epidermis was determined by counting the number of mucous cells along the epidermis between the lateral line canals and dividing by the length of epidermis underlying this distance, as measured along the epidermal basement membrane.

Epidermal thickness was estimated by placing a grid with parallel randomly spaced lines on the video monitor, then the shortest distance was measured between the intersection of each line with the epidermal basement membrane to the surface of the fish ($n > 45$ measurements/fish). Arithmetic and harmonic mean thickness were calculated from these measurements. The harmonic mean thickness estimates diffusion capacity and the arithmetic mean thickness estimates tissue mass (Weibel 1979).

Statistics

One-way analysis of variance (ANOVA) (alpha = 0.05) was used with Tukey's post hoc comparisons (alpha = 0.05) to determine treatment level effects of pH 6.3, 5.1 and 4.3 at 8 mg Ca/L and 0 μg Al/L (Nie et al. 1975). Variances of the treatment groups were all homogeneous (Hartley's F-max test, $p \geqslant 0.01$, Milliken and Johnson 1984).

Differences in epidermal structure resulting from exposure were also tested by three-way ANOVA using the BMDP statistical package (Dixon et al. 1985). The following main factors effects were tested: pH (5.1, 4.3), calcium (0.5, 8.0 mg/L),

TABLE 1. Mean (standard error of the mean) epidermal thickness (μm) and mucous cell diameter (μm), mucous cell number, and mucous cell volume density (%) for fish exposed to specified combinations of pH, calcium (mg/L), and aluminum (μg/L).

pH	Ca	Al	*n*	Arithmetic epidermal thickness	Harmonic mucous cell diameter	Mucous cell diameter	Mucous cells/mm	Volume density mucous cells
6.3	0.5	0	2	19.0(0.38)	18.5(0.33)	15.1(1.21)	36.4(1.74)	27.6(3.35)
6.3	8.0	0	5	18.7(1.48)	18.3(1.53)	13.5(0.37)	16.8(2.05)	11.8(2.63)
5.1	0.5	0	5	17.7(0.81)	16.7(0.84)	13.9(0.31)	32.7(1.44)	26.5(1.74)
5.1	0.5	37	5	18.7(0.86)	17.9(0.81)	14.3(0.48)	35.8(2.41)	26.5(1.42)
5.1	0.5	111	5	23.8(2.74)	22.7(2.43)	14.2(0.46)	30.4(1.96)	16.8(2.47)
5.1	8.0	0	5	17.2(1.15)	16.9(1.13)	13.9(0.68)	19.2(0.84)	18.7(1.38)
5.1	8.0	37	5	17.1(0.87)	16.3(0.97)	13.8(0.38)	23.7(1.76)	21.4(2.09)
5.1	8.0	111	5	22.6(2.50)	18.6(1.09)	13.6(0.30)	21.6(2.03)	15.6(2.46)
4.3	0.5	0	3	23.0(2.85)	22.0(2.67)	16.3(1.20)	40.0(4.68)	27.2(1.37)
4.3	0.5	37	5	19.1(0.94)	18.5(2.07)	14.8(0.23)	37.6(4.09)	29.5(1.64)
4.3	0.5	111	4	16.5(1.96)	16.1(1.93)	13.7(0.92)	26.3(5.70)	23.8(3.93)
4.3	8.0	0	5	21.6(1.07)	21.2(1.06)	16.0(0.57)	31.2(3.91)	27.5(2.54)
4.3	8.0	37	5	21.2(1.45)	20.6(1.94)	15.6(0.53)	31.6(4.65)	22.6(2.66)
4.3	8.0	111	5	22.1(2.09)	21.3(1.86)	14.6(0.59)	31.4(3.28)	26.6(1.80)

and aluminum (0, 37, 111 μg/L). Since aluminum was not an experimental variable at pH 6.3, fish sampled at this pH were not included in the three-way ANOVA. When there were no significant two- or three-way (higher-order) interactions, significant main factor effects of pH and calcium (pH 5.1 vs. 4.3; 0.5 vs. 8.0 mg Ca/L) were determined by the *F*-test at alpha = 0.01. Comparisons of main factor effects for aluminum (0 vs. 37 vs. 111 μg/L) and for all pairwise comparisons of treatment means were determined by the Scheffe multiple comparison procedure at alpha = 0.01. Where significant higher-order interactions occurred, such as pH × Al, factor means were compared with the Scheffe multiple comparison procedure adjusted for interactions (Neter et al. 1985).

If the three-way interaction was not significant, the corresponding sum of squares was pooled with the error sum of squares to interpret two-way interactions and main factor effects. If neither the two-way nor three-way interactions were significant, the corresponding sums of squares were pooled with the error sum of squares to interpret main factor effects (Neter et al. 1985). A significance level of alpha = 0.01 was used for the seven ANOVA comparisons (pH, Ca, Al, pH × Ca, pH × Al, Ca × Al, pH × Ca × Al). This assured that if no effects were actually present, there would be no greater than a 6.8% chance of one or more of the seven comparisons leading to the conclusion of an effect (Kimball inequality, Neter et al. 1985).

Results

Qualitative histological observations of the epidermis indicated an increase in both size and number of mucous cells with exposure to low levels of pH 4.3 (Fig. 1). However, it was difficult to qualitatively determine these effects at pH 5.1. In addition, the number of mucous cells appeared to be elevated with exposure to 0.5 mg Ca/L and the epidermis tended to be thicker with exposure to elevated aluminum concentrations, but we could not definitively interpret these effects qualitatively.

1. Effects of pH: One-Way Analysis of Variance

Results from the quantitative stereological measurements for mucous cell volume density, mucous cell diameter, mucous cell number, and epidermal thickness of fish exposed to various combinations of pH, calcium, and aluminum exposure are listed in Table 1. At 8.0 mg Ca/L and 0 μg Al/L, fish exposed to pH 4.3 and 5.1 had mucous cells that were significantly more numerous, larger, and had a greater volume density than fish exposed to pH 6.3. In addition, mucous cell number, size, and volume density were also significantly increased with exposure to pH 4.3 relative to pH 5.1. Neither the harmonic nor arithmetic mean epidermal thickness were significantly effected by pH.

2. Combined Effects of pH, Aluminum, and Calcium: Three-Way Analysis of Variance

No significant differences occurred between treatment means for mucous cell volume density, number, diameter, or epidermal thickness. However, both pH (main factor effects, averaged for all calcium and aluminum exposures) and calcium (main factor effects, averaged for all pH and aluminum exposures) significantly effected epidermal mucous cell diameter or number (Table 2). The complete three-way analysis of variance tables are presented in Ingersoll (1986).

Low pH significantly increased mucous cell volume density, diameter and number/mm (Table 2). Mucous volume density at pH 4.3 (26.2%) was about 25% greater than at pH 5.1 (20.9%) when averaged for all calcium and aluminum exposures. This increase in mucous cell volume density at pH 4.3 resulted from a hypertrophy of mucous cells. An 8.6% increase in average mucous cell diameter at pH 4.3 (15.2 μm) compared to pH 5.1 (14.0 μm) is an increase of about 25% in cell volume, assuming mucous cells are spherical. In addition, mucous cell number for fish exposed to pH 4.3 (33.0 cells/mm) was about 21% greater than at pH 5.1 (27.2 cells/mm) when averaged for all calcium and aluminum exposures. Thus, the increase in mucous cell volume density at pH 4.3 resulted from both a hypertrophy and hyperplasia of mucous cells.

Low calcium significantly increased the number of mucous cells/mm (Table 2). Mucous cell number for fish exposed to 0.5 mg Ca/L (33.8 cells/mm) was about 28% greater than at 8.0 mg Ca/L (26.4 cells/mm) when averaged for all pH and aluminum exposures.

Neither pH, calcium, nor aluminum exposure significantly effected harmonic mean epidermal thickness (Table 2). The only significant effect of exposure on arithmetic mean epidermal thickness was an interaction between pH and aluminum. This interaction resulted from epidermal thickness increasing at pH 5.1 and decreasing at pH 4.3 with exposure to increasing aluminum concentrations.

Discussion

Exposure of brook trout to toxic combinations of pH and calcium that affected survival, weight, or hatching (Ingersoll et al. 1985) were accompanied by either mucous cell hypertrophy or hyperplasia and were most pronounced at pH 4.3 or 0.5 mg Ca/L. These morphological changes reportedly occur in Atlantic salmon (*Salmo salar*, Daye and Garside 1980) and brown bullhead (*Ictalurus nebulosus*, Zuchelkowski et al. 1981, 1986) exposed to low pH alone. In addition, both increased mucous cell number and epidermal thickness have been observed in threespine sticklebacks (*Gasterosteus aculeatus*) exposed to low calcium alone (Wendelaar–Bogna 1978). Epithelial lifting, cytolysis, necrosis, or rupturing of mucous cells reportedly occur with acute exposure to acid or aluminum (brook trout, *Salvelinus fontinalis;* Daye and Garside 1976; Tandjung 1982; brown trout, *Salmo trutta*: Segner et al. 1988). However, in our study and studies by Zuchelkowski et al. (1981, 1986) these effects were not observed. These differences may be attributed to the fact that fish in these previous studies were exposed to much more acutely toxic acid or aluminum stress in hard water.

Survival of brook trout fry was reduced only at aluminum concentrations greater than 333 μg/L and survival generally improved at intermediate aluminum exposure concentrations of 111–333 μg/L (Ingersoll et al. 1985). Concurrently, epidermal structure of fish surviving exposure to 37–111 μg Al/L was not consistently affected. Perhaps if fish had been sampled from the more severe aluminum exposures (333–1000 μg Al/L), effects on epidermal structure might have been observed. Additionally, after prolonged exposure to ionoregulatory stress, epidermal mucous cell differentiation may decline (Agarwal et al. 1979). Perhaps if fish had been sampled over time during the 40 d exposure, more pronounced effects on epidermal structure might have been observed.

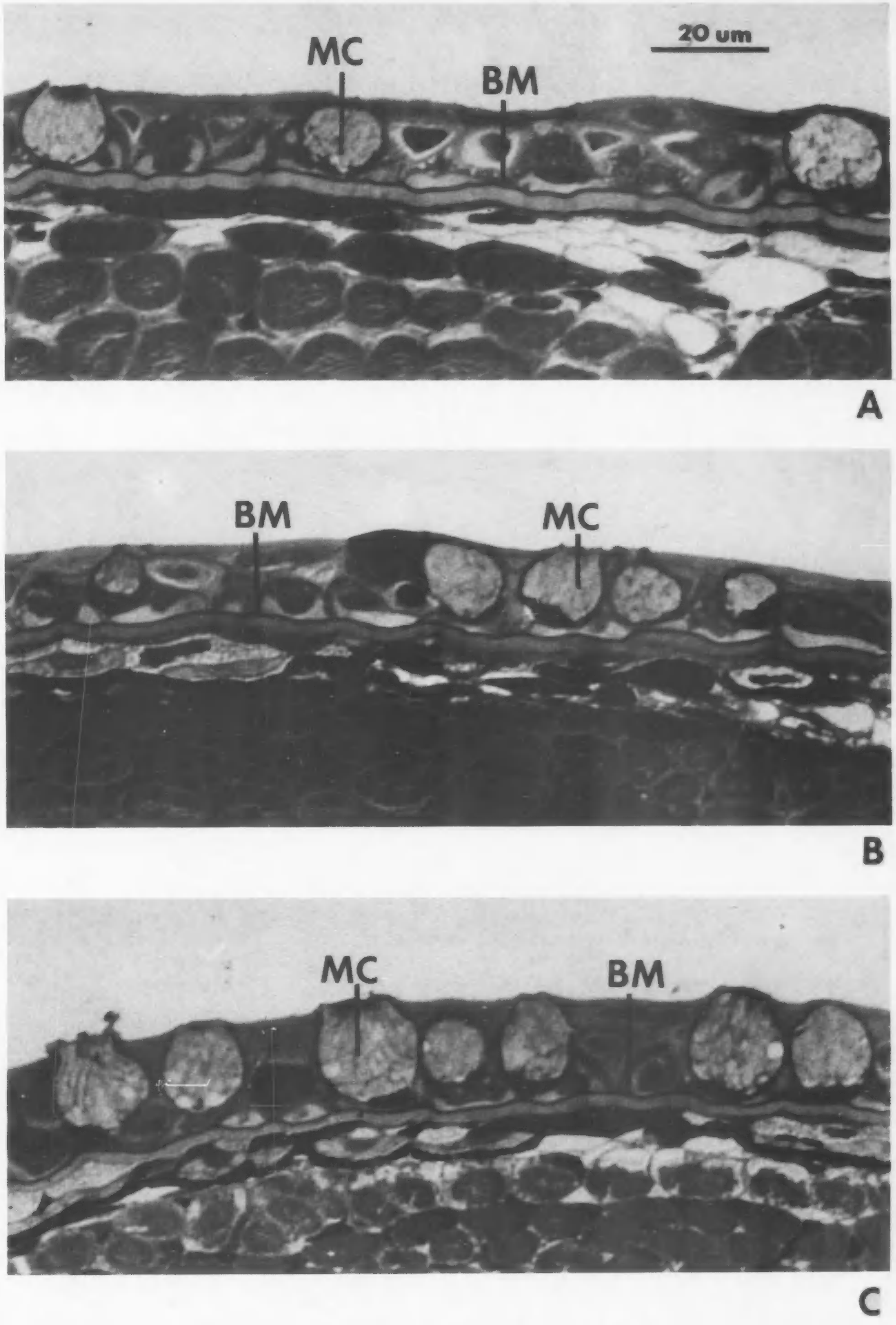
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FIG. 1. Representative cross sections of epidermis sampled from fry exposed to pH 6.3 (A), 5.1 (B), and 4.3 (C) at 8.0 mg Ca/L and 0 μg Al/L. MC = mucous cell, BM = basement membrane.

TABLE 2. Summary of three-way analysis of variance on the effect of pH, calcium (mg/L), and aluminum (μg/L) on fish epidermis.

	Main factor effect means[a]							Two-way interactions			Three-way interactions
	pH		Ca		Al			pH × Ca	pH × Al	Ca × Al	pH × Ca × Al
Parameter	5.1	4.3	8.0	0.5	0	37	111				
Mucous cell volume density (%)	20.9	26.2	22.1	25.1	25.0	25.0	20.7	NS[b]	NS	NS	NS
Mucous cell diameter (μm)	14.0	15.2	14.6	14.5	15.0	14.6	14.0	NS	NS	NS	NS
Number of mucous cells/mm	27.2	33.0	26.4	33.8	30.8	32.2	27.4	NS	NS	NS	NS
Harmonic mean epidermal thickness (μm)	18.2	20.0	19.1	19.0	19.2	18.3	19.7	NS	NS	NS	NS
Arithmetic mean epidermal thickness (μm)	19.5	20.6	20.3	19.8	19.9	19.0	21.3	NS	*[c]	NS	NS

[a] Underlined main factor effect means are significantly different.

[b] Not significant.

[c] Significant two-way interaction between pH and Al, so main factor effects of these two variables cannot be interpreted from the ANOVA table.

In subsequent studies (Wood et al. 1990a, b), we found reductions in whole body ions of brook trout fry exposed to similar combinations of pH and calcium affecting epidermal mucous cells in the present study. Fish exposed to acidic conditions often experience increased mucus production (Dively et al. 1977; Miller and Mackay 1982) and ionoregulatory stress (Fromm 1980; Spry et al. 1981; Leivestad 1982). The epithelial layer of the gill, gut, kidney, urinary bladder, and skin may be important in ionoregulation in fish (Johnson 1973; Marshall 1977; Marshall and Bern 1979). Of these organs, only the skin is well developed just after hatching (Shelbourne 1957; Morgan 1974; Marshall and Bern 1980). Newly hatched fry of brook trout (McCay et al. 1936), rainbow trout, brown trout (Parry 1961), salmon (Hayes et al. 1946; Peterson and Martin–Robichaud 1986), and larvae of several marine fish (Shelbourne 1957; Lasker and Threadgold 1958) can regulate calcium and sodium before their gills are well developed. With ionoregulatory stress, the skin of fish may either thicken (Roberts et al. 1970), develop chloride cells (Roberts et al. 1973; Leonard and Summers 1976; Lasker and Threadgold 1968), or the number of epidermal mucous cells may increase (Mattheij et al. 1972; Leonard and Summers 1976). While we did not see a consistent effect on epidermal thickness or chloride cells, the hypertrophy and hyperplasia of epidermal mucous cells and increased mucus production were probably more subtle responses of brook trout fry to ionoregulatory stress resulting from exposure to either low pH or low calcium.

The exact role of mucus in ionoregulation requires further clarification (Van de Winkle et al. 1986). Removal of the mucus coating from the skin of various teleosts disturbs salt or freshwater tolerance (e.g. Pickford et al. 1966). Yet, in vitro, mucus may not impede ion diffusion. The diffusion coefficient of mucus isolated from the Pacific staghorn sculpin (*Leptocottus armatus*) was similar to control saline solution (Marshall 1978). However, in vivo, preparations of black flounder (*Rhombosolea retiaria*) esophagus epithelium maintained a gradient of calcium, sodium, and potassium activity in an unstirred layer 50–1200 μm above the mucus coating. This gradient may restrict ion movement by either increasing resistance of the epidermis to diffusion or decreasing mixing and convection at the surface of the skin (Shephard 1981, 1982).

Mucus supports an external polyanionic charged layer that may aid in the transfer of ions across the epithelium. This charged layer may raise the concentration of cations at the surface of the skin close to the internal body concentrations, even at low cation concentrations in the surrounding enviornment (Bennett 1963; Philpot 1968; Kirschner 1978; Crowther and Marriott 1984; Van de Winkle et al. 1986; Whitear 1986). Hence, this layer could either decrease loss of cations across the body surface or help to concentrate cations from ion deficient water. However, increased acidity may titrate these negatively charged residues, thus interfering with the ion binding properties of mucus (McDonald 1983). Additional research is needed to better determine the role of mucus and mucous cells in ionoregulation of fish exposed to acidic or neutral levels of pH.

In conclusion, the changes observed in epidermal mucous cells may be a compensatory mechanism used by brook trout fry to counter ionoregulatory stress resulting from extended exposure to either low pH or low calcium. Furthermore, the use of high resolution light microscopy, image analysis, and stereological techniques allowed for a quantitative determination of subtle morphological changes in the epidermis of fry that could not be achieved using qualitative histological techniques.

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References

Agarwal, S. K., A. K. Rai, T. K. Banerjee, and A. K. Mittal. 1979. Histophysiology of the Epidermal Mucous Cells in Relation to Salinity in a Fresh-Water Teleost *Heteropneustes fossilis* (Bloch) (Heteropneusidae, Pisces). Zool. Beitr. 25: 403–410.

Aherne, W. A., and M. S. Dunnill. 1982. Morphometry. Edward Arnold Ltd., London, UK. 205 p.

Baker, J. P. 1984. Fishes, p. 5–74 to 5–139. *In* A. A. Altshuller and R. A. Linthurst [ed.] The acid deposition phenomenon and its effects; critical assessment review papers. Vol. II. Effects Sciences. EPA-600/8-83-016B. U.S. Environmental Protection Agency. Washington, DC.

Bennett, H. S. 1963. Morphological aspects of extracellular polysaccharides. J. Histochem. Cytochem. 11: 14–23.

Bergman, H. L., J. S. Mattice, and D. J. A. Brown. 1988. Lake acidification and fisheries project: adult brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1561–1562.

Crowther, R. S., and C. Marriott. 1984. Counter-ion binding to mucus glycoproteins. J. Pharm. Pharmacol. 36: 21–26.

Daye, P. G., and E. T. Garside. 1976. Histopathologic changes in surfical tissues of brook trout, *Salvelinus fontinalis* (Mitchill), exposed to acute and chronic levels of pH. Can. J. Zool. 54: 2140–2155.

1980. Structural alterations in embryos and alevins of the Atlantic salmon, *Salmo salar* L., induced by continuous or short-term exposure to acidic levels of pH. Can. J. Zool. 58: 27–43.

DeHoff, R. T., and F. N. Rhines. 1968. Quantitative microscopy. McGraw-Hill, New York, NY. 422 p.

Dively, J. L., J. E. Mudge, W. H. Neff, and A. Anthony. 1977. Blood PO_2, PCO_2, and pH changes in brook trout (*Salvelinus fontinalis*) exposed to sublethal levels of acidity. Comp. Biochem. Physiol. 57A: 347–351.

Dixon, W. J., M. B. Brown, L. Engelman, J. W. Frane, M. A. Hill, R. I. Jennrich, and T. D. Toporek. 1985. BMDP statistical software. University of California Press, Berkeley, CA. 734 p.

Elias, H., A. Henning, and D. E. Schwartz. 1971. Stereology: applications to biomedical research. Physiol. Rev. 51: 158–200.

Fromm, P. O. 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Environ. Biol. Fishes 5: 79–93.

Gunn, J. M., and W. Keller. 1984. Spawning site water chemistry and lake trout (*Salvelinus namaycush*) sac fry survival during spring snowmelt. Can. J. Fish. Aquat. Sci. 41: 319–329.

Harris, J., and S. Hunt. 1973. Epithelial mucins of the Atlantic salmon (*Salmo salar* L.). Biochem. Soc. Trans. 1: 153–155.

Hayes, F. R., D. A. Darcy, and C. M. Sullivan. 1946. Changes in the inorganic constituents of developing salmon eggs. J. Biol. Chem. 163: 621–631.

Ingersoll, C. G. 1986. The effects of pH, aluminum, and calcium on survival and growth of brook trout (*Salvelinus fontinalis*) early life stages. PhD. thesis. University of Wyoming, Laramie, WY. 122 p.

Ingersoll, C. G., T. W. La Point, H. L. Bergman, and J. Breck. 1985. An early life stage brook trout (*Salvelinus fontinalis*) experiment to determine the effects of pH, calcium, and aluminum in low conductivity water, p. 42–48. *In* P. J. Rago and R. K. Schreiber [ed.] Symp. Proc., 114th Ann. Meet., Am. Fish. Soc., Biol. Rep. 80 (40.21): 106 p.

Ingersoll, C. G., D. R. Mount, D. Gulley, T. W. La Point, and H. L. Bergman. 1990. Effects of pH, aluminum, and calcium on survival and growth of eggs and fry of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 1580–1592.

Johnson, D. W. 1973. Endocrine control of hydromineral balance in teleosts. Am. Zool. 13: 799–818.

Kelso, J. R. M., and C. K. Minns. 1982. Current status of lake acidification and its effect on the fishery resources of Canada, p. 69–81. *In* R. Johnson [ed.] Acid Rain/Fisheries. Northeastern Div. Am. Fish. Soc. Bethesda, MD.

Kirschner, L. B. 1978. External charged layer and Na^+ regulation, p. 310–324. *In* Alfred Benzon symposium XI. Osmotic and volume regulation. Academic Press, New York, NY.

Lasker, R., and L. T. Threadgold. 1968. "Chloride cells" in the skin of the larval sardine. Exp. Cell Res. 52: 582–590.

Leivestad, H. 1982. Physiological effects of acid stress on fish, p. 157–164. *In* R. Johnson [ed.] Acid Rain/Fisheries. Northeastern Div. Am. Fish. Soc. Bethesda, MD.

Leonard, J. B., and R. G. Summers. 1976. The ultrastructure of the integument of the American eel, *Anguilla rostrata*. Cell Tissue Res. 171: 1–30.

Marshall, W. S. 1977. Transepithelial potential and short-circuit current across the isolate skin of *Gillichthys mirabilis* (Teleostei: Godiidae), acclimated to 5% and 100% seawater. J. Comp. Physiol. 114: 157–165.

1978. On the involvement of mucous secretion in teleost osmoregulation. Can. J. Zool. 56: 1088–1091.

Marshall, W. S., and H. A. Bern. 1979. Teleostean urophysis: urotensin II and ion transport across the isolated skin of a marine teleost. Science (Wash., DC) 204: 519–521.

1980. Ion transport across the isolated skin of the teleost, *Gillichthys mirabilis*, p. 337–350. *In* B. Lahlou [ed..] Epithelial transport in the lower vertebrates. Proceedings of the memorial symposium to Jean Maetz, Station Zoologique of Villefranche-sur-Mer, France, 26–27 June 1978.

Mattheij, J. A. M., H. W. J. Stroband, F. J. Kingma, and P. G. W. J. Van Oordt. 1972. Prolactin and osmoregulation in the cichlid fish, *Cichlasoma biocellatum*, and the effect of this hormone on the thyroid, gills, and skin. Gen. Comp. Endocrin. 18: 607.

McCay, C. M., A. V. Tunison, M. Crowell, and H. Paul. 1936. The calcium and phosphorus content of the body of the brook trout in relation to age, growth, and food. J. Biol. Chem. 114: 259–263.

McDonald, D. G. 1983. The effects of H^+ upon the gills of freshwater fish. Can. J. Zool. 61: 691–703.

Miller, T. G., and W. C. Mackay. 1982. Relationship of secreted mucus to copper and acid toxicity in rainbow trout. Bull. Environ. Contam. Toxicol. 28: 68–74.

Milliken, G. A., and D. E. Johnson. 1984. Analysis of messy data. Volume I: Designed experiments. Lifetime learning publications, London, UK. 473 p.

Mo,rgan, M. 1974. Development of secondary lamellae of the gills of the trout, *Salmo gairdneri* (Richardson). Cell Tissue Res. 151: 509–523.

Muniz, I. P., and H. Leivestad. 1980. Acidification-effects on freshwater fish, p. 84–92. *In* D. Drablos and A. Tollan [ed.] Ecological impact of acid precipitation: proceedings of an international conference. Sandefjord, Norway. March 11–14, 1980. SNSF-project, As-NLH, Norway.

Neter, J., W. Wasserman, and M. H. Kutner. 1985. Applied linear statistical models. Richard D. Irwin Inc., Homewood, IL. 1127 p.

Nie, N. H., C. H. Hull, J. G. Jenkins, K. Steinbrenner, and D. H. Bent. 1975. SPSS Statistical package for the social sciences. McGraw-Hill, New York, NY. 675 p.

Parry, G. 1961. Osmotic and ionic changes in blood and muscle of migrating salmonids. J. Exp. Biol. 38: 411–427.

Peterson, R. H., and D. J. Martin–Robichaud. 1986. Growth and major inorganic cation budgets of Atlantic salmon alevins at three ambient acidities. Trans. Am. Fish. Soc. 115: 220–226.

Philpot, C. W. 1968. The functional implications of the cell surface: the plasmalemma and surface-associated polyanions, p. 109–122. *In* R. Porter and M. O'Conner [ed.] Cystic fibrosis. CIBA Foundation study group 32. Churchill, London, UK.

Pickford, G. E., P. K. T. Pang, and W. H. Sawyer. 1966. Prolactin and serum osmolality of hypophysectomized killifish, *Fundulus heteroclitus*, in fresh-water. Nature (Lond.) 209: 1040–1041.

Roberts, R. J., M. Bell, and H. Young. 1973. Studies on the skin of plaice (*Pleuronectes platessa* L.). II. The development of larval plaice skin. J. Fish Biol. 5: 103–108.

Roberts, R. J., W. M. Shearer, K. G. R. Elson, and A. L. S. Munro. 1970. Studies on ulcerative dermal necrosis of salmonids. 1. The skin of the normal salmon head. J. Fish Biol. 2: 223–229.

Rombough, P. J. 1982. Effects of low pH on eyed embryos and alevins of Pacific salmon. Can. J. Fish. Aquat. Sci. 40: 1575–1582.

Schofield, C. L. 1982. Historical fisheries changes in the United States related to decreases in surface water pH, p. 57–67. *In* R. Johnson [ed.] Acid Rain/Fisheries. Northeastern Div. Am. Fish. Soc. Bethesda, MD.

Segner, H., M. Linnenbach, and R. Marthaler. 1979. Towards a use of epidermal mucous cells in the field assessment of acid stress in fish. J. Appl. Ichthyol. 3: 187–190.

Segner, H., R. Marthaler, and M. Linnenbach. 1988. Growth, aluminum uptake and mucous cell morphometrics of early life stages of brown trout, *Salmo trutta*, in low pH water. Envir. Biol. Fish. 21: 153–159.

Shelbourne, J. E. 1957. Site of chloride regulation in marine fish larvae. Nature (Lond.) 180: 920–922.

Shephard, K. L. 1981. The influence of mucus on the diffusion of water across fish epidermis. Physiol. Zool. 54: 224–229.

1982. The influence of mucus on the diffusion of ions across the esophagus of fish. Physiol. Zool. 55: 23–34.

Spry, D. J., C. M. Wood, and P. V. Hodson. 1981. The effects of environmental acid on freshwater fish with particular reference to the softwater lakes in Ontario and the modifying effects of heavy metals. A literature review. Can. Tech. Rep. Fish. Aquat. Sci. 999: 145 p.

Tandjung, S. D. 1982. The acute toxicity and histopathology of brook trout (*Salvelinus fontinalis*, Mitchill) exposed to aluminum in acid water. PhD dissertation. Fordham University, New York, NY. 213 p.

Tietge, J. E., R. D. Johnson, and H. L. Bergman. 1988. Morphometric changes in gill secondary lamellae of brook trout (*Salvelinus fontinalis*) after long-term exposure to acid and aluminum. Can. J. Fish. Aquat. Sci. 45: 1643–1648.

Van de Winkle, J. G. J., T. H. M. S. M. van Kuppevelt, H. M. J. Janssen, and R. A. C. Lock. 1986. Gylcosaminoglycans in the skin mucus of rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 85B: 473–475.

Van Oosten, J. 1957. The skin and scales, p. 207–244. *In* M. E. Brown [ed.] The physiology of fishes. Vol. 1. Metabolism. Academic Press, New York, NY.

Weibel, E. R. 1979. Stereological methods vol. 1. Practical methods for biological morphometry. Academic press, New York, NY. 415 p.

Wendelaar-Bogna, S. E. 1978. The effects of changes in external sodium, calcium, and magnesium concentrations on prolactin cells, skin, and plasma electrolytes of *Gasterosteus aculeatus*. Gen. Comp. Endocrin. 34: 265–275.

Whitear, M. 1986. Epidermis. Chapter 2, p. 8–38. *In* J. Bereiter–Hahn, A. G. Matoltsy, and K. S. Richards [ed.] Biology of the Integument 2. Vertebrates. Springer-Verlag, New York, NY.

Wood, C. M., D. G. McDonald, C. G. Ingersoll, D. R. Mount, O. E. Johannsson, S. Landsburger, and H. L. Bergman. 1990. Effects of water acidity, calcium, and aluminum on whole body ions of brook trout (*Salvelinus fontinalis*) continuously exposed from fertilization to swim-up: a study by instrumental neutron activation analysis. Can. J. Fish. Aquat. Sci. 47: 1593–1603.

1990b. Whole body ions of brook trout (*Salvelinus fontinalis*) alevins: responses of yolk-sac and swim-up stages to water acidity, calcium, and aluminum, and recovery effects. Can. J. Fish. Aquat. Sci. 47:1604–1615.

Wright, R., and E. Snekvik. 1978. Acid precipitation: chemistry and fish populations in 700 lakes in southernmost Norway. Verh. Int. Vere. Limnol. 20: 765–775.

Zuchelkowski, E. M., R. C. Lantz, and D. E. Hinton. 1981. Effects of acid-stress on epidermal mucous cells of the brown bullhead *Ictalurus nebulosus* (LeSeur): A morphometric study. Anat. Rec. 200: 33–39.

1986. Skin mucous cell response to acid stress in male and female brown bullhead catfish, *Ictalurus nebulosus* (LeSeur). Aquat. Tox. 8: 139–148.

Responses of Brook Trout (*Salvelinus fontinalis*) Fry to Fluctuating Acid, Aluminum, and Low Calcium Exposure

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Brook trout fry were exposed for thirty days to temporal combinations of two water qualities: (1) pH 5.21,51 μg/L total aluminum, with 2.39 mg/L calcium (baseline); and (2) pH 4.59, 329 μg/L total aluminum, with1.56 mg/L calcium (pulse). Although continuous exposure to the latter conditions caused nearly complete mortality, exposures alternating between the two conditions caused much less mortality. In fact, fish experiencing 18 d of baseline conditions before their first pulse exposure had mortality rates equal to fish never experiencing a pulse, indicating acclimation to acid/aluminum/low calcium stress. We conclude that the pattern of fluctuating acid/aluminum/low calcium exposure is very important in determining its toxicity, and that the toxicity of acidic "pulses" in field situations may be lower than would be expected from laboratory exposures using naive fish, due to the existence of acclimation.

Des alevins d'omble de fontaine ont été exposés pendant trente jours à des combinaisons d'eau de deux qualités: (1) pH 5,21, 51 ug d'aluminium total par litre (L) avec 2,39 mg/L de calcium (conditions de base); et (2) pH 4,59, 329 μg/L d'aluminium total avec 1,56 mg/L de calcium (exposition brève à une teneur élevée). Bien qu'une exposition continue aux dernières conditions ait tué presque tous les alevins, des expositions alternées aux deux types d'eau ont provoqué une mortalité beaucoup moindre. En fait, le taux de mortalité des poissons exposés pendant 18 jours aux conditions de base avant leur première exposition à une teneur élevée était égal à celui des poissons qui n'avaient jamais été exposés à une "poussée", ce qui montre une adaptation à une agression acide-aluminium-faible calcium. Nous concluons que le profil de l'exposition fluctuante acide-aluminium-faible calcium est très important pour déterminer sa toxicité, et que la toxicité des "poussées" acides sur le terrain peut être plus faible qu'on s'y attendrait à partir des expositions expérimentales de poissons naïfs à cause de l'existence du phénomène d'adaptation.

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Precipitation and snowpack sampling have shown that atmospheric deposition of acid is at least partially responsible for increased acidity of surface waters in some areas (Galloway et al. 1983; LaZerte and Dillon 1984). While long-term acidification has been documented in many watersheds, episodic inputs of atmospheric acids (e.g. snowmelt or heavy rainfall events) to surface waters may cause more severe, short-term depressions in pH, or "pulses" (Jeffries et al. 1979). Especially sensitive are systems with high flushing rates, such as streams and rivers, although lentic systems may also experience pulses (e.g. Gunn and Keller 1984).

Concurrent with the depression of surface water pH are increases in concentrations of several metals, particularly aluminum. Aluminum is a very common constituent in minerals, and its solubility is strongly pH dependent. Consequently, acidic waters leach increased amounts of aluminum from watershed minerals, resulting in increased aluminum concentrations in surface waters. Although specific water concentrations of aluminum vary among watersheds, elevated aluminum concentrations can be toxic to fish, and are believed to be a major toxic factor in some areas (Driscoll et al. 1980; Baker 1982). Finally, dilution of stream or lake waters by runoff can cause calcium concentrations to decrease during pulses; this may be an important change as well, as calcium has been shown to reduce acid/aluminum toxicity (Brown 1983; Ingersoll et al. 1990a).

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In certain instances, fish kills have been directly associated with acidic pulses. In the Adirondack region of New York, brook trout (*Salvelinus fontinalis*) held in Little Moose Lake water over winter died suddenly and precipitously during spring runoff (Schofield and Trojnar 1980). Annual fish kills have been observed in Plastic Lake, Ontario (Harvey and McArdle 1986); large fish kills have been well documented in Norwegian rivers as well (Leivestad and Muniz 1976; Hesthagen 1986). During in situ exposures in Ontario, Gunn and Keller (1984) observed the greatest mortality of lake trout (*Salvelinus namaycush*) sac fry during a period of increased acid and aluminum concentrations during snowmelt. Nonetheless, losses of fish populations have often been attributed to more gradual or seasonal acidification rather than short-term, catastrophic events. But even in cases of apparently gradual acidification, undetected episodes may occur, since the timing and frequency of water chemistry sampling may preclude observation of a pulse.

Results from exposures of fish to fluctuating concentrations of various toxicants show that mortality during such pulsed exposures frequently cannot be extrapolated from exposures to constant toxicant concentrations (e.g. Curtis et al. 1985; Seim et al. 1984; but see Breck 1988). This limits our ability to extrapolate much of the available laboratory toxicity data to field situations in which fluctuating exposures may occur, and makes clear the need for a better understanding of fish responses to these exposures. As the discussion above suggests, the need for a better understanding is particularly acute in the study of lake acidification, where pulsed exposures are known to occur.

Despite the potential importance of pulsed acid/aluminum exposures, responses of fish to such conditions have only recently received attention in the laboratory (Siddens et al. 1986; Klauda and Palmer 1987). Design of relevant pulse exposures in the laboratory can be difficult, due to the tremendous variety of pulses that can occur in the field (Jeffries et al. 1979; Gunn and Keller 1986). Replicating an observed field episode provides some site-specific information, but has limited utility in predicing responses to pulses of different characteristics. Alternatively, controlled laboratory exposures, which can isolate responses to single variables such as exposure frequency, timing, duration, peak concentrations, and rise/fall times, seem a prudent first step toward understanding the effect of acid/aluminum pulses on fish. The present study focuses on the frequency and timing of pulses, as they affect the responses of brook trout during fluctuating exposure to acid, aluminum, and low calcium.

Materials and Methods

Test water and Exposure System

Test water was reconstituted from well water purified by reverse osmosis and deionization treatment as described by Mount et al. (1988a), except that iron oxidation/precipitation, acidification (to reduce carbonate concentrations), and forced-draft deaeration (to remove excess CO_2) replaced sodium chloride softening in the water treatment process. Two 190-L, flow-through headtanks were continuously supplied with this water and maintained at pH 5.2 and 4.6, respectively, by automatic pH controllers (Leeds and Northrup #7380), adding dilute H_2SO_4. Function of these pH controllers was continuously monitored and integrated with the laboratory alarm system. Aluminum and calcium were added as chloride stock solutions, delivered directly into the headtanks via Mariotte bottles. These test waters were then delivered to modified proportional dilution systems (after Mount and Brungs 1967) functioning solely as flow splitters. Test chambers were 800 mL glass aquaria, each receiving 25 mL/min of test water (45 volume additions/day).

Experimental Animals

Test fish were brook trout (*Salvelinus fontinalis*) fry of a Temiscamie × domestic hybrid strain, obtained as eggs from Brandon Enterprises, Paul Smiths, NY. Prior to testing, eggs and fry were reared in soft water (nominally, pH 6.5, 2 mg/L calcium, 1 mg/L sodium). Fry were 35 d (about 400 degree-days) past the swim-up stage, weighing 97.0 ± 26.2 mg (wet weight; mean + SD) when the exposure began. Fish were fed trout starter (Rangen's, Inc., Buhl, ID) ad libitum twice daily during rearing and exposure.

Experimental Design

Exposure treatments were temporal combinations of two water qualities (Table 1): (1) pH 5.21, 51 μg/L total aluminum (21 μg/L inorganic monomeric), with 2.39 mg/L calcium (baseline); and (2) pH 4.59, 329 μg/L total aluminum (244 μg/L inorganic monomeric), with 1.56 mg/L calcium (pulse). These acid, aluminum, and calcium concentrations were chosen to represent water quality changes during a theoretical episodic event in the Adirondack region of New York (based on Schofield 1982). Treatments were based on five consecutive, 6-d periods (30 d total) of either baseline (B) or pulse (P) conditions, named according to the nature of the treatment: PBBPB (i.e. 6 d of pulse, 12 d of baseline, 6 d of pulse, 6 d of baseline); BPBPB, BBPPB, BBBPB, and a "baseline conrol" (BBBBB). In addition, we included a "pulse control," a 21-d exposure to pulse conditions (followed by 9 d of baseline), to both assess the toxicity of constant exposure to pulse conditions, and enable comparison of the sensitivity of these brook trout fry to results obtained in 21-d exposures by Ingersoll et al. (1990a) and Parkhurst et al. (1990). Each treatment was composed of ten replicate chambers, with 25 fish in each chamber.

Test Procedures

At the initiation of the experiment (30 April 1986), all chambers were receiving baseline chemistry water. Twenty-five fish were transferred from rearing water to exposure chambers in groups of five fish. As soon as all fish were in the exposure system, the PBBPB and pulse control treatments were changed to pulse chemistry. These and all similar changes in water chemistry were accomplished by gradually changing the inflowing water to the appropriate diluters from 250 mL/min baseline water to 250 mL/min pulse water, via five 50 mL/min changes in the flow of each test water, at 1-h intervals (i.e. 250 mL/min baseline to 200 mL/min baseline and 50 mL/min pulse, etc.). Changes from pulse to baseline conditions were achieved by the reverse procedure. This gradual change (about 6 h) was intended to prevent possible mortalities due to extremely rapid changes in water chemistry. Figure 1 shows the changes in pH and monomeric aluminum concentration, measured in an actual exposure chamber, during the initiation of a representative pulse.

Mortality, pH (Sargent-Welch model LS; Orion Ross combination electrode), temperature, and toxicant stock solution

TABLE 1. Mean, standard error of the mean, and sample size of all measured chemical parameters, for both baseline and pulse conditions. All units are mg/L unless otherwise noted.

	Baseline		Pulse	
Parameter	Mean ± SEM	*n*	Mean ± SEM	*n*
pH (units)	5.21 ± <0.01	234	4.59 ± <0.01	126
Calcium	2.39 ± 0.06	28	1.56 ± 0.03	8
Total Al (μg/L)	51.4 ± 2.8	26	329.0 ± 14.0	12
Monomeric Al (μg/L)	44.3 ± 2.0	26	310.0 ± 11.0	12
Inorganic Al (μg/L)	21.1 ± 1.7	26	244.0 ± 17.0	12
Sodium	1.35 ± 0.06	28	1.10 ± 0.01	8
Potassium	1.23 ± 0.20	28	1.90 ± 0.34	8
Magnesium	0.89 ± 0.02	28	0.77 ± 0.01	8
Chloride	2.94 ± 0.05	30	2.97 ± 0.07	17
Sulfate	10.0 ± 0.3	30	11.2 ± 0.5	17
Fluoride (μg/L)	32.5 ± 2.5	28	30.1 ± 3.4	17
Nitrate	0.19 ± 0.03	30	0.36 ± 0.04	17
Dissolved Oxygen	8.15 ± 0.04	36	8.33 ± 0.05	12
Total Ammonia	<0.1[a]	—	<0.1[a]	—
Temperature (C)	11.5 ± <0.1	234	11.2 ± 0.01	126
Conductivity (μS/cm)	39.7 ± 0.8	46	43.1 ± 0.7	38

[a] All ammonia concentrations were less than 0.1 mg/L, too low for accurate determinations by our methods.

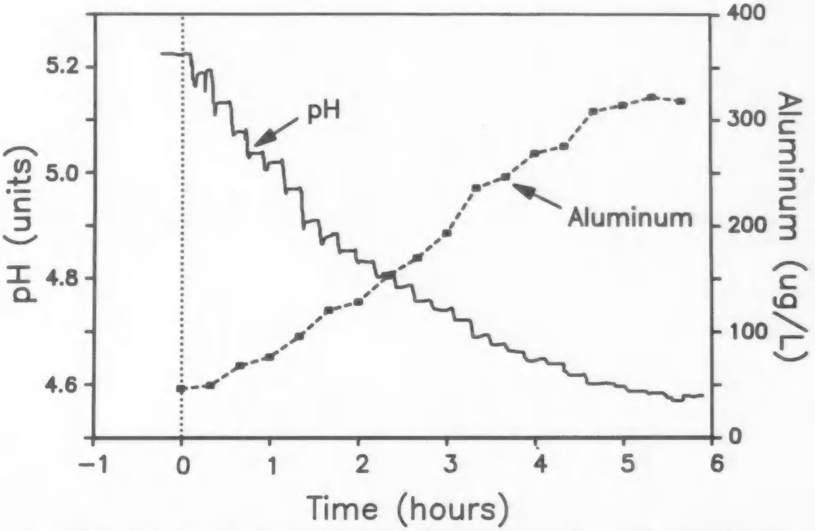

FIG. 1. Changes in pH (solid line) and monomeric aluminum concentration (dashed line) measured in an exposure chamber during a change from baseline to pulse conditions. Adjustments to the inflowing water were made at 0, 1, 2, 3, and 4 h. Changes in pH were continuously recorded from a pH probe submersed in the exposure chamber; monomeric aluminum was measured in samples taken from the exposure chamber at 20-min intervals.

flow were monitored daily; during changes in water chemistry, pH was measured hourly. Dissolved oxygen (Yellow Springs Instruments #54 ARC), ammonia (Orion #407A meter and #9512 probe), and conductivity (Extech #440) were measured periodically. Exposure chambers were siphoned daily to remove particulate matter. At the termination of the exposure, the number and wet weight of all surviving fish were recorded.

Once during each of the five 6-d periods, water samples for chemical analysis were drawn from each of two replicate chambers for each treatment. Cations (aluminum, calcium, sodium, potassium, and magnesium) were measured by atomic absorption spectroscopy (Perkin-Elmer, model #2380), using flame or high-temperature graphite furnace (Perkin-Elmer, model HGA-400) and standard methods (USEPA 1979). Aluminum was fractionated into total, monomeric (fast-reactive), and inorganic monomeric (dialysis/fast-reactive) after the method of LaZerte (1984). Anions (chloride, fluoride, sulfate, and nitrate) were measured by anion chromatography (Dionex #2110; AS-4 column).

Statistics

Survival data (after arcsine/square root transformation) and growth data were analyzed by one-way analysis of variance.

Homogeneity of variances and normality were verified by the Hartley, Bartlett, and chi-square tests ($P=0.01$; USEPA 1985; Neter et al. 1985). The Tukey procedure was used for post hoc comparison of means ($P=0.05$; Zar 1984). To compare responses of fish in each treatment during the first 6 d of pulse exposure, mortality during the period was calculated by subtracting the percent cumulative mortality immediately before experiencing the pulse conditions from the percent mortality after the first 6 d of pulse exposure. Mortality during the second 6 d of pulse exposure, and during the first 12 d of continuous pulse exposure (BBPPB and Pulse control only) were calculated similarly.

In addition to these parametric procedures, treatment groups were also compared by testing the similarity of their survival curves. The LIFETEST procedure in SAS (SAS 1985) computes nonparametric estimates of the survival distribution using individual times to death. The homogeneity of the survival estimates over treatment groups was evaluated using the logrank test (SAS 1985), which places more weight on later survival times, and the Wilcoxon test (SAS 1985), which places more weight on early survival times.

Results

Baseline chemistry represented a moderate stress for these brook trout, causing 30.3% mortality, most of which occurred before day 18 (Fig. 2). In contrast, pulse chemistry was highly stressful, causing 96.2% mortality, but again occurring predominately before day 18 (Fig. 2). Toxicity of both baseline and pulse conditions was similar to that observed by Ingersoll et al. (1990a) and Parkhurst et al. (1990).

Qualitatively, responses to the four remaining treatments (PBBPB, BPBPB, BBPPB, and BBBPB) varied according to the timing or duration of the pulse(s) (Fig. 2). In the PBBPB treatment, mortality rate rose above the baseline control rate 48–72 h into the initial pulse (test day 2–3), and remained high until about day 12, even though exposure conditions returned to baseline on day 6. Interestingly, mortality rate was not affected by the second pulse on days 18–24, and remained low for the remainder of the test. The BPBPB treatment showed a similar response; mortality rate increased after 48–72 h at pulse conditions (day 8–9), remained high for 6 d after cessation of the initial pulse, and showed little response to the second pulse. The response was somewhat altered after 12 d of baseline exposure in the BBPPB treatment. Here, mortality rate did not increase as sharply upon initiation of the first pulse. By the end of the exposure, however, total mortality was not different from that seen in both PBBPB and BPBPB treatments (Table 2).

The most marked change in response was observed in the BBBPB treatment. In this treatment, mortality rate was similar to that in the baseline control treatment throughout the exposure. There was no apparent response to the pulse during days 18–24, even though this was the first pulse experienced by these fish. Mortality in the BBBPB group at the end of the experiment was not different from that in baseline control group, and was significantly lower than in any other treatment group (Table 2). Furthermore, the BBBPB and baseline control treatments had the only two mortality curves which could not be differentiated by the LIFETEST procedure (Table 2).

Differential responses during specific periods of pulse exposure were clearly indicated by both ANOVA and LIFETEST analyses of mortality (Table 2). Mortality during the first 6 d of pulse exposure was similar in the pulse control, PBBPB, BPBPB, and BBPPB treatment groups, but was significantly lower in the BBBPB treatment group. Mortality during the second 6 d of pulse exposure was highest in the pulse control group, intermediate in the BBPPB group, and quite low in both PBBPB and BPBPB groups.

Only the BBPPB and pulse control treatments included 12 d of continuous exposure to pulse conditions. However, exposure to 12 d of baseline conditions prior to 12 d of pulse clearly affected the response to the pulse; mortality during the 12 d of pulse was significantly different between the BBPPB and pulse control treatments (Table 2).

Mean weight at the end of the exposure was lowest in the pulse control group, but no significant differences were found among any treatment groups (Table 2).

Dicussion

The results of this experiment clearly indicate that the sensitivity of these brook trout fry to acid/aluminum exposure changed during the course of the exposure. While exposure to the pulse conditions was quite toxic during the first 12 d of the experiment, fry exposed to baseline conditions of 18 d prior to experiencing pulse conditions showed no observable response to the pulse. There are two potential explanations for this change in sensitivity: (1) physiological changes associated with growth and development of the fry; or (2) acclimation to acid/aluminum exposure.

Although developmental changes cannot be unequivocally excluded, we believe that they are not the cause for the observed change in sensitivity. The fry used in this exposure had absorbed all of their yolk reserves prior to the exposure, and had been feeding exogenously for several weeks; fry at this stage have well-formed gills, and have completed most of the rapid development characteristic of the alevin stage. Furthermore, the ability of fish to acclimate to aluminum has been demonstrated (Orr et al. 1986; Wood et al. 1988a, b). Perhaps most convincing, however, is the observance of acclimation to aluminum over a similar time period (10–15 d) in older, fingerling brook trout (McDonald et al. 1990). We feel, therefore, that the most probable explanation is physiological changes which increased resistance to the more severe exposure. For the purposes of this discussion, such changes will be referred to as acclimation.

Increased resistance to acid, aluminum, and low calcium exposure may occur via multiple mechanisms. Several researchers have shown that fish native to acidic waters are less sensitive to more severe acid or acid/aluminum challenges (Rahel 1983; Mcwilliams 1980; Brown 1981). For example, Rahel (1983) found that yellow perch from acidic lakes were more resistant to low pH challenge than those from more neutral lakes, but sublethal pre-exposure of fish from neutral lakes to low pH did not increase their resistance. As Rahel surmised, this suggests that genetic selection (adaptation), rather than acclimation, was responsible for the observed differences in resistance. In the present study, selection processes (i.e. mortality of the most sensitive individuals) cannot explain the lack of mortality during pulse exposure in the BBBPB treatment. Although some mortality occurred in the BBBPB treatment prior to pulse exposure, cumulative mortality after the 18 d of baseline exposure and 6 d of pulse exposure was only 25%; in contrast, cumulative mortality after only 12 d of baseline exposure and 6 d of pulse exposure was 36–61% in the PBBPB, BPBPB, and BBPPB treatments. Therefore, if selection were the operative mechanism, one would expect a minimum of 11–

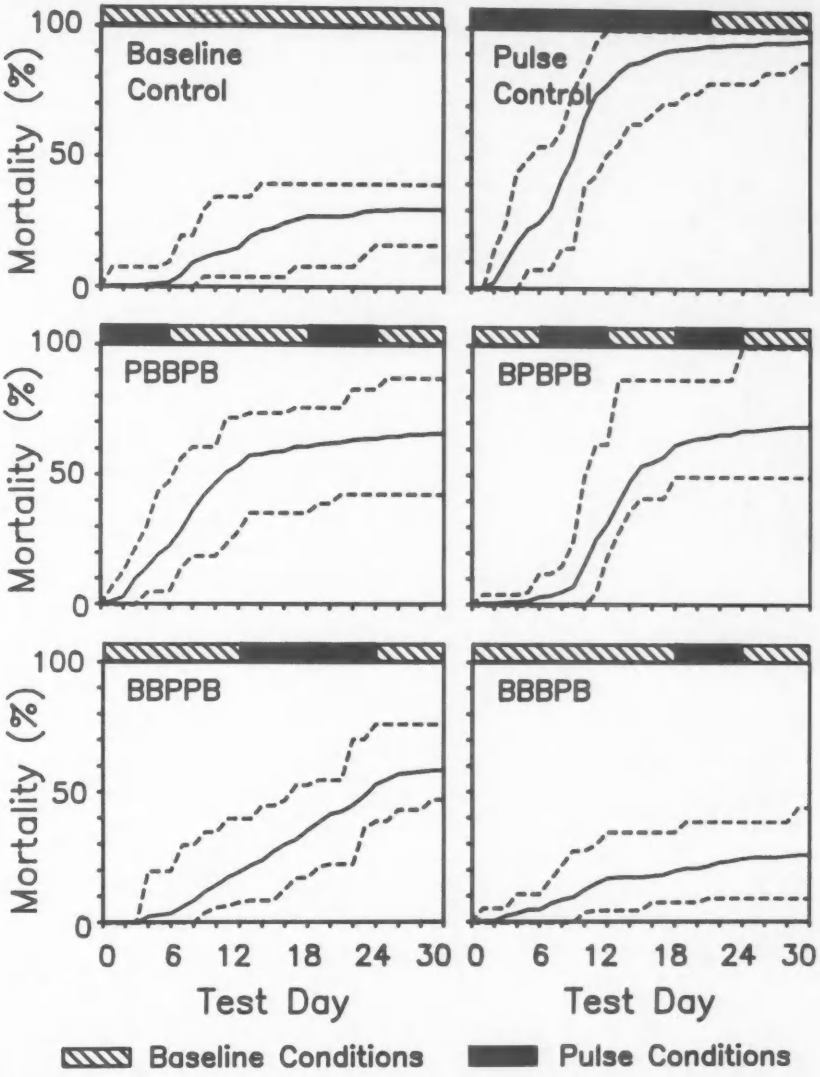

FIG. 2. Cumulative mortality in each of the six treatments. Solid lines represent mean mortality across all replicates; the area between the dashed lines represents the actual range of values from the ten replicate chambers. Shaded bars along the top of each graph depict the experimental design. Cross-hatched bars indicate days at baseline conditions while solid areas indicate days at pulse conditions. Measured chemistry for these conditions is given in Table 1.

37% more mortality in the BBBPB treatment than was observed. This, combined with literature evidence for acclimation, leads us to conclude that selection processes were not responsible for the observed change in sensitivity to pulse conditions.

Recently, the physiological changes responsible for acclimation of brook trout to acid/aluminum stress have been demonstrated (Wood et al. 1988a, b; McDonald and Milligan 1988). The response of naive fish to acid/aluminum exposure has been characterized as occurring in two phases (Booth et al. 1988): (1) the "shock" phase (0–48 h), identified by massive efflux of sodium and chloride, and inhibition of sodium uptake at the gill; and (2) the "recovery" phase (>48 h), during which efflux is reduced, despite continued inhibition of uptake. Some respiratory disturbances may accompany these ioneregulatory-disturbances (Wood et al. 1988c). After 10 wk of sublethal pre-exposure to aluminum, however, the response to more severe acid/aluminum challenge is altered. Compared to the response of naive fish, pre-exposed fish showed no signs of respiratory disturbance, and perhaps more importantly, showed a marked acceleration of the recovery phase, with much more rapid re-establishment of ionic equilibrium (Wood et al. 1988a, b). This reduced ionoregulatory disturbance appears linked to increased sodium transport capacity in fish pre-exposed to aluminum (McDonald and Milligan 1988). Interestingly, pre-exposure to acid alone did not increase resistance to acid/aluminum chal-

TABLE 2. Summary of results from analysis of variance and mean comparisons. Different letters designate statistically separable groups. Note that the ANOVA compares the amount of mortality during the specified period, while the LIFETEST procedure compares the pattern of mortality.

Endpoint	Treatment	Mean[a] ± SEM[b]	ANOVA grouping	LIFETEST grouping
30-d mortality (%)	BBBPB	26.0 ± 3.8	A	A
	Baseline control	30.3 ± 2.3	A	A
	BBPPB	58.5 ± 3.3	B	B
	PBBPB	66.1 ± 4.2	B	C
	BPBPB	69.0 ± 5.4	B	D
	Pulse control	96.2 ± 1.0	C	E
Mortality during first 6 d at pulse conditions (%)	BBBPB	4.8 ± 1.8	A	A
	BBPPB	16.9 ± 3.2	B	B
	PBBPB	22.2 ± 4.1	B	B
	Pulse control	25.9 ± 5.2	B	B
	BPBPB	30.8 ± 4.2	B	B
Mortality during second 6 d at pulse conditions (%)	PBBPB	2.9 ± 2.8	A	A
	BPBPB	6.0 ± 9.9	A	A
	BBPPB	16.9 ± 3.9	B	B
	Pulse control	53.3 ± 6.9	C	C
Mortality during first 12 consecutive days at pulse conditions (%)	BBPPB	33.8 ± 3.6	A	A
	Pulse control	79.2 ± 5.0	B	B
Mean weight at end of exposure (mg)	Baseline control	151 ± 4.0	A	—[c]
	BPBPB	149 ± 4.0	A	—
	BBBPB	145 ± 4.0	A	—
	BBPPB	144 ± 4.0	A	—
	PBBPB	140 ± 6.0	A	—
	Pulse control	130 ± 13.0	A	—

[a] Mean survival was determined by pooling the ten replicates.

[b] Standard errors for survival data were approximated from arcsine/square root transformed data.

[c] Not applicable.

lenge. While these findings define the physiological nature of aluminum acclimation, they provide no clues as to the time-course of the acclimatory process. Our data suggest that 12–18 d are necessary for full acclimation to develop. Some degree of acclimation was evident after 12 d of baseline exposure, as mortality in the BBPPB treatment during the first 12 d of pulse conditions was significantly lower than that seen in the pulse control group (Table 2). However, only a full 18 d of exposure to baseline conditions (BBBPB) was effective in eliminating mortality due to pulse conditions. The time course of this acclimation is similar to that observed in other studies in our laboratory, and seems compatible with the mechanism outlined above.

Neither food consumption nor feeding behavior were quantified during the exposure. Nevertheless, casual observation suggested that feeding behavior was depressed by the initiation of the experiment, but increased markedly during days 12–18 of the experiment in all treatments; feeding behavior did not seem to decrease during subsequent pulse exposure (days 18–24) in the PBBPB, BPBPB, BBPPB, and BBBPB treatments. Similar cessation and resumption of feeding during acid/aluminum stress has been reported for juvenile brook trout (Ingersoll et al. 1990b). Moreover, Mount et al. (1988b) showed a definite correlation between osmoregulatory disturbance and reduced growth/feeding in adult brook trout during acid, aluminum, and low calcium exposure. As resumption of feeding in our study seemed to coincide with the observed increase in aluminum tolerance, we hypothesize that the resumption of feeding may result from the accommodation of initial ionoregulatory disturbances, leading to increased aluminum tolerance. The fact that these changes in feeding behavior were observed in all treatments, even the pulse control, may be related to the lack of significant effects on growth. Without quantitative data, however, no definitive conclusions can be drawn regarding the potential relationship between feeding behavior and acid/aluminum acclimation.

Orr et al. (1986) demonstrated aluminum acclimation in rainbow trout after only 7 d of pre-exposure, and further showed no increase in tolerance with 14 or 21 d of pre-exposure. While differences in experimental designs complicate comparisons with our study, it seems clear that, had we used a similar design, 7 d would not have been sufficient to show complete acclimation for brook trout, and further, that aluminum tolerance in brook trout (if tested similarly) would show an increase over time. The reasons for this apparent inter-specific difference, if it does exist, are not clear.

Siddens et al. (1986) found that intermittent exposure to acid/aluminum could be more stressful than continuous exposure to mean or even peak concentrations of acid/aluminum. Such a finding is not entirely supported by the present study, since we found that the pattern of intermittent exposure can alter the sensitivity of brook trout to acid/aluminum stress. Siddens et al. (1986) made no mention of any acclimatory phenomena, although their study was not designed to delineate such effects. In addition, the low stress condition used in that study was of relatively high pH (7.3) with no added aluminum. This may be responsible, in part, for the difference in results, given that changes from low stress to high stress conditions in the Siddens study represented a quite large (and possibly unrealistic for natural conditions) alteration of water chemistry. Furthermore, acclimation to metals derived from sub-lethal pre-exposure can be lost when fish are returned to clean water (Bradley et al. 1985; Dixon and Sprague 1981). The absence of aluminum in Siddens' low-stress condition may have prevented acclimation to aluminum, and therefore, increased the stress of the intermittent exposure.

Guthrie (1982) was successful in reducing acid- and acid/aluminum-induced mortality of brook trout by introducing acid and aluminum over the course of 5 d, as opposed to an instantaneous introduction. This finding probably represents yet another acclimatory phenomenon. Our results indicate that this 5-d period would be insufficient for brook trout to acquire full resistance to aluminum from the pre-exposure. Instead, we believe that Guthrie's approach alleviates stress only from extremely rapid changes in acid/aluminum concentrations. More gradual introduction of acid and aluminum may allow fish to pass from the "shock" phase into the "recovery" phase (about 48 h) before exposure conditions become severe enough to cause massive mortality. Further evidence for this alternative mechanism stems from Guthrie's success in acclimating fish to low pH alone, while the work of McDonald and Milligan (1988) suggests that long-term pre-exposure to low pH does not increase resistance to further reductions in pH. In our study, acid and aluminum were always introduced over the same period (about 6 h), indicating actual resistance to a more severe exposure, rather than reduced stress from rapidly changing concentrations.

Increased acid/aluminum resistance of all three types (genetic selection, long-term physiological acclimation, gradual changes in water chemistry) have important applications to the study and/or mitigation of surface water acidification. First, the existence of genetic selection suggests that laboratory bioassays using hatchery or otherwise less adapted strains of fish may overestimate the toxicity of acid and/or aluminum to fishes native to acidic waters. Second, long-term physiological acclimation to aluminum suggests that acid/aluminum pulses associated with snowmelt or storm events may be less important than would be predicted from exposures beginning with naive fish. Since fish populations subject to pulses would likely be chronically exposed to lower levels of aluminum, tolerance of pulses such as that seen in the present study can be expected in many natural populations. This may further imply that long-term increases in acidification can be more important to the survival of fish populations than are pulses. At a minimum, our data indicate that for increased relevance to field situations, future laboratory studies on the effect of fluctuating acid, aluminum, and calcium concentrations should be conducted after a 2–3 wk sub-lethal pre-exposure, to permit acclimation. Finally, gradual introduction of acid, aluminum, and low calcium to naive strains of fish could certainly reduce mortality during stocking of acidified waters.

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References

Baker, J. P. 1982. Effects on fish of metals associated with acidification,p. 165–176. *In* R. E. Johnson [ed.] Acid rain/fisheries. Proceedings of an international symposium on acidic precipitation and fishery impacts in northeastern North America. Am. Fish. Soc., Bethesda, MD.

Booth, C. E., D. G. McDonald, B. P. Simons, and C. M. Wood. 1988. The effects of aluminum and low pH on net ion fluxes and ion balance in the brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1563–1574.

Bradley, R. W., C. DuQuesnay, and J. B. Sprague. 1985. Acclimation of rainbow trout, *Salmo gairdneri* Richardson, to zinc: kinetics and mechanism of enhanced tolerance induction. J. Fish. Biol. 27: 367–379.

Breck, J. E. 1988. Relationships among models for acute toxic effects: applications to fluctuating concentrations. Environ. Contam. Toxicol. 7: 775–778.

Brown, D. J. A. 1981. The effects of various cations on the survival of brown trout, *Salmo trutta* at low pHs;. J. Fish. Biol. 18: 31–40.

1983. Effect of calcium and aluminum concentrations on the survival of brown trout (*Salmo trutta*) at low pH. Bull. Environ. Contam. Toxicol. 30: 582–587.

Curtis, L. R., W. K. Seim, and G. A. Chapman. 1985. Toxicity of fenvalerate to developing steelhead trout following continuous or intermittent exposure. J. Toxicol. Environ. Health 15: 445–457.

Dixon, D. G., and J. B. Sprague. 1981. Acclimation to copper by rainbow trout (*Salmo gairdneri*) — a modifying factor in toxicity. Can. J. Fish. Aquat. Sci. 38: 880–888.

Driscoll, C. T., J. P. Baker, J. J. Bisogni, and C. L. Schofield. 1980. Effect of aluminum speciation on fish in dilute acidified waters. Nature (Lond.) 284: 161–164.

Galloway, J. N., C. L. Schofield, N. E. Peters, G. R. Hendrey, andE. R. Altwicker. 1983. Effect of atmospheric sulfur on the composition of three Adirondack lakes. Can. J. Fish. Aquat. Sci. 40: 799–806.

Gunn, J. M., and W. Keller. 1984. Spawning site water chemistry and lake trout (*Salvelinus fontinalis*) sac fry survival during spring snowmelt. Can. J. Fish. Aquat. Sci. 41: 319–329.

1986. Effects of acid meltwater on chemical conditions at nearshore spawning sites. Water Air Soil Pollut. 30: 545–552.

Guthrie, C. A. 1982. Acclimation of brook trout (*Salvelinus fontinalis*) to acidified waters. M.S. thesis, Cornell University, Ithaca, NY. 134 p.

Harvey, H. H., and J. M. McArdle. 1986. Physiological responses of rainbow trout *Salmo gairdneri* exposed to Plastic Lake inlet and outlet stream waters. Water Air Soil Pollut. 30: 687–694.

Hesthagen, T. 1986. Fish kills of Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) in an acidified river of SW Norway. Water Air Soil Pollut. 30: 619–628.

Ingersoll, C. G., D. D. Gulley, D. R. Mount, J. D. Fernandez, J. R. Hockett, M. E. Mueller, and H. L. Bergman. 1990b. Aluminum and acid toxicity to two strains of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 1641–1648.

Ingersoll, C. G., D. R. Mount, D. D. Gulley, T. W. LaPoint, and H. L. Bergman. 1990a. Effects of pH, aluminum, and calcium on survival and growth of brook trout embryos (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 1580–1592.

Jeffries, D. S., C. M. Cox, and P. J. Dillon. 1979. Depression of pH in lakes and streams in central Ontario during snowmelt. J. Fish. Res. Board Can. 36: 640–646.

Klauda, R. J., and R. E. Palmer. 1987. Responses of blueback herring eggs and larvae to pulses of acid and aluminum. Trans. Am. Fish. Soc. 116: 561–569.

LaZerte, B. D. 1984. Forms of aqueous aluminum in acidified catchments of central Ontario: a methodological analysis. Can. J. Fish. Aquat. Sci. 41: 766–776.

LaZerte, B. D., and P. J. Dillon. 1984. Relative importance of anthropogenic versus natural sources of acidity in lakes and streams of central Ontario. Can. J. Fish. Aquat. Sci. 41: 1664–1677.

Leivestad, H., and I. P. Muniz. 1976. Fish kill at low pH in a Norwegian river. Nature (Lond.) 259: 391–392.

McDonald, D. G., C. M. Wood, R. G. Rhem, M. E. Mueller, D. R. Mount, and H. L. Bergman. 1990. Nature and time course of acclimation to aluminum in juvenile brook trout (*Salvelinus fontinalis*). I. Physiology. Can. J. Fish. Aquat. Sci. (In press)

McDonald, D. G., and C. L. Milligan. 1988. Sodium transport in the brook trout, *Salvelinus fontinalis*: the effects of prolonged low pH exposure in the presence and absence of aluminum. Can. J. Fish. Aquat. Sci. 45: 1606–1613.

McWilliams, P. G. 1980. Effects of pH on sodium uptake in Norwegian brown trout (*Salmo trutta*). J. Exp. Biol. 88: 259–267.

Mount, D. I., and W. A. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. Water Res. 1: 21–29.

Mount, D. R., C. G. Ingersoll, D. D. Gulley, J. D. Fernandez, T. W. LaPoint, and H. L. Bergman. 1988a. Effect of long-term exposure to acid, aluminum and calcium on adult brook trout (*Salvelinus fontinalis*): 1. Survival, growth, fecundity, and progeny survival. Can. J. Fish. Aquat. Sci. 45: 1623–1632.

Mount, D. R., J. R. Hockett, and W. A. Gern. 1988b. Effect of long-term exposure to acid, aluminum, and low calcium on adult brook trout (*Salvelinus fontinalis*): 2. Vitellogenesis and osmoregulation. Can. J. Fish. Aquat. Sci. 45: 1633–1642.

Neter, J., W. Wasserman, and M. H. Kutner. 1985. Applied linear statistical models. Richard D. Irwin, Inc., Homewood, IL. 1119 p.

Orr, P. L., R. W. Bradley, J. B. Sprague, and N. J. Hutchinson. 1986. Acclimation-induced change in toxicity of aluminum to rainbow trout (*Salmo gairdneri*). Can. J. Fish. Aquat. Sci. 43: 243–246.

Parkhurst, B. R., H. L. Bergman, J. D. Fernandez, D. D. Gulley, J. R. Hockett, and D. A. Sanchez. 1990. Inorganic monomeric aluminum and pH as predictors of acidic water toxicity to brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 1631–1640.

Rahel, F. J. 1983. Population differences in acid tolerance between yellow perch, *Perca flavescens*, from naturally acidic and alkaline lakes. Can. J. Zool. 61: 147–152.

SAS Institute Inc. 1985. SAS user's guide: statistics, version 5 ed. SAS Institute Inc., Cary, NC. 956 p.

Schofield, C. L. 1982. Historical fisheries changes in the United States related to decreases in water pH, p. 57–67. *In* R.E. Johnson [ed.] Acid rain/fisheries. Proceedings of an international symposium on acidic precipitation and fishery impacts in northeastern North America. Am. Fish. Soc., Bethesda, MD.

Schofield, C. L., and J. R. Trojnar, 1980. Aluminum toxicity to brook trout (*Salvelinus fontinalis*) in acidified waters, p. 341–366. *In* T. Toribara, M. Miller, and P. Morrow [ed.] Polluted Rain. Plenum Press, New York, NY.

Seim, W. K., L. R. Curtis, S. W. Glenn, and G. A. Chapman. 1984. Growth and survival of developing steelhead trout (*Salmo gairdneri*) continuously or intermittently exposed to copper. Can. J. Fish. Aqua. Sci. 41: 433–438.

Siddens, L. K., W. K. Seim, L. R. Curtis, and G. A. Chapman. 1986. Comparison of continuous and episodic exposure to acidic, aluminum-contaminated waters of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 43: 2036–2040.

United States Environmental Protection Agency (USEPA). 1979. Methods for chemical analysis of water and wastes. EPA-600/4-79-020, Environmental Protection Agency, Washington, DC.

United States Environmental Protection Agency (USEPA). 1985. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, Environmental Protection Agency, Cincinatti, OH.

Wood, C. M., D. G. McDonald, C. E. Booth, B. P. Simons, C. G. Ingersoll, and H. L. Bergman. 1988a. Physiological evidence of acclimation to acid/aluminum stress in adult brook trout (*Salvelinus fontinalis*).1. Blood composition and net sodium fluxes. Can. J. Fish. Aquat. Sci. 45: 1587–1596.

Wood, C. M., B. P. Simons, D. R. Mount, and H. L. Bergman. 1988b. Physiological evidence of acclimation to acid/aluminum stress in adult brook trout (*Salvelinus fontinalis*). 2. Blood parameters by cannulation. Can. J. Fish. Aquat. Sci. 45: 1597–1605.

Wood, C. M., R. C. Playle, B. P. Simons, G. G. Goss, and D. G. McDonald. 1988c. Blood gases, acid-base status, ions, and hematology in adult brook trout (*Salvelinus fontinalis*) under acid/aluminum exposure. Can. J. Fish. Aquat. Sci. 45: 1575–1586.

Zar, J. H. 1984. Biostatistical analysis. Prentice-Hall, Englewood Cliffs, NJ. 718 p.

Inorganic Monomeric Aluminum and pH as Predictors of Acidic Water Toxicity to Brook Trout (*Salvelinus fontinalis*)

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Inorganic monomeric aluminum concentrations were found to be the primary determinant of survival of brook trout feeding fry exposed for 21 d to various combinations of total aluminum and pH, plus several concentrations of fluoride or DOC, or several temperatures. Total aluminum concentrations in the 4 × 4 × 4 experimental matrices ranged from 3–1276 ppb, pH from 4.4–6.7, fluoride from <10–328 ppb, DOC from <1–9.2 ppm, and temperatures from 5.4–12.0°C. Stepwise logistic regression was used to determine the significance of the effect of each water quality variable on survival. Inorganic monomeric aluminum accounted for 78–98% of the variation in survival. Except in the fluoride bioassay where pH was not significant, pH was the second-most important variable, accounting for up to 16% of the variation in survival. Fluoride or temperature accounted for 1–2% of the variation in survival, while DOC accounted for 6% of the variation in survival.

Des chercheurs ont trouvé que des concentrations de composés inorganiques monomériques à base d'aluminium étaient le principal facteur de survie des alevins d'omble de fontaine en phase d'alimentation exposés pendant 21 jours à différentes combinaisons d'aluminium total et de pH, en plus de plusieurs concentrations de fluorure, de COD, ou à plusieurs températures. Les concentrations d'aluminum total dans les matrices expérimentales 4 × 4 × 4 étaient comprises entre 3 et 1276 parties par milliard, le pH entre 4,4 et 6,7, les fluorures, de moins de 10 parties par milliard à 328 parties par milliard, le COD, de moins de 1 à 9,2 ppm, et les températures de 5,4 à 12,0 °C. Une régression logistique par étape a été utilisée pour déterminer l'importance de l'effet de chaque variable de la qualité de l'eau sur la survie. Les composés inorganiques monomériques à base d'aluminium représentaient de 78 à 98% de la variation au niveau de la survie. À l'exception des dosages biologiques des fluorures où le pH n'était pas important, le pH était la deuxième variable en importance, comptant jusqu'à 16% de la variation au niveau de la survie. Les fluorures ou la température représentait de 1 à 2% de la variation tandis que le carbone organique dissous (COD) représentait 6% de la variation de la survie.

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Research on the effects of surface water acidification on fish has shown that water quality exerts complex influences on fish responses to acidification (Baker 1983; Martin 1987). Since acidity (pH) is a primary controlling and regulating variable in many chemical, biological, and geological processes, changes in pH cause many additional changes in water quality, all of which can potentially affect aquatic organisms.

Of primary importance are the effects of pH on the solubility and speciation of toxic trace metals, especially aluminum, which is abundant in most aquatic environments. The solubility of aluminum trihydroxide increases exponentially below about pH 6 (Drever 1982). Consequently, pH and aluminum concentrations are highly correlated in acidic surface waters (Driscoll 1983). Aluminum has also been shown to be toxic in acidic surface waters (Baker and Schofield 1982).

A number of studies have investigated the toxic effects of aluminum and acidity on fish (Baker and Schofield 1982; Cleveland et al. 1986; Dalziel et al. 1986; Fivelstad and Leivestad 1984; Gunn and Noakes 1986; Gunn and Noakes 1987; Hunn et al. 1987; Hutchinson and Holtze 1985; Ingersoll et al. 1990; Mount et al. 1988; Mount et al. 1990; Muniz and Leivestad 1980; Rosseland and Skogheim 1984; Schofield and Trojnar 1980; Siddens et al. 1986; Skogheim and Rosseland 1984). Most of these were clean water, laboratory studies in which the only water quality variables were pH and aluminum. Studies of the effects of water quality variables on aluminum toxicity to fish in acidic waters have mostly beeen limited to studies of calcium (Bergman et al. 1988; Brown 1981; Dalziel et al. 1986; Ingersoll et al. 1990), Mount et al. 1990; Wood et al. 1990a, b), although Baker (1981) did some limited work on the effects of fluoride and DOC on aluminum toxicity, and Gunn (1986) has looked at some of the effects of water temperature. Not well understood are the effects of aluminum speciation on aluminum toxicity, which have only been investigated by Driscoll et al. (1980).

Aluminum toxicity depends on the concentrations and species of aluminum present (Baker 1981). The toxic species of aluminum are considered to be inorganic monomeric species such as Al^{3+} and ionic Al-OH complexes (Baker 1983). Chemical ligands that complex these species may reduce their bioavailability and toxicity. In acidic waters, the most common aluminum complexes are aluminum-fluoride and organic aluminum compounds, which comprise a majority of the total aluminum present (Driscoll et al. 1987). Baker (1981) found that 500 ppb of fluoride significantly reduced aluminum toxicity to brook trout fry at pH 4.4 and 5.2, but not at pH 4.0. However, the significance of this conclusion is not clear, since most waters sensitive to acidification have fluoride concentrations much less than 500 ppb. In contrast, Clark and LaZerte (1985) found that fluoride-aluminum complexes did not have a significant effect on the toxicity of acidic aluminum solutions to amphibian eggs and tadpoles. Baker (1981) also found that the addition of DOC (as citrate) to acidic aluminum solutions virtually eliminated aluminum toxicity.

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Both acid and aluminum toxicity are also affected by water temperature (Gunn 1986). Temperature influences rates of most chemical and biological processes. Increased temperature is often associated with increased mortality rates for organisms exposed to toxic chemicals, since uptake rates increase as metabolic rates increase. Temperature also influences solubilities of chemicals. In acidic waters, the solubility of amorphous $Al(OH)_3$ increases as temperature decreases (Siep et al. 1984).

The objective of this research was to determine whether inorganic monomeric aluminum and pH were the best water quality measurements for predicting the effects of acidic waters on the survival of brook trout fry over a wide range of DOC and fluoride concentrations and temperatures in the laboratory. The hypothesis tested was that the best predictors of acidic water toxicity to brook trout are pH and the concentration of inorganic monomeric aluminum. Alternative hypotheses would be that the best predictors of toxicity are measurements of (1) pH alone, (2) inorganic monomeric aluminum alone, (3) pH and other measurements of aluminum such as total aluminum, or (4) pH, total aluminum and the concentrations of ligands that affect aluminum toxicity and speciation, such as fluoride, DOC, temperature, calcium, etc. For our hypothesis, DOC, fluoride and temperature were considered to be factors that may change the toxicity of the total concentration of aluminum present by changing its speciation and solubility.

Methods

Biology

All of the bioassays were done at the Red Buttes Environmental Biology Laboratory, University of Wyoming, Laramie, WY. Experimental fish were Temiscamie-domestic hybrid strain brook trout (*Salvelinus fontinalis*). Fertilized eggs approximately 48-h old were obtained from Brandon Enterprises, Paul Smiths, NY, in late October 1985. The parental brood stock was from circumneutral softwater lakes in Adirondack Park, NY. The three bioassays described below were run sequentially during February, March, and April, 1986, on brook trout feeding fry of approximately the same size and from the same stock of eggs. To accomplish this, when the eggs were received from Brandon Enterprises they were divided into four egg incubation trays. Each tray was incubated at a different temperature, 2, 5, 8, or 12°C, to stagger developmental rates and hatching times. Thus, fish at the same life stage and approximate size, from the same stock of eggs, but not of the same chronological age, were used in each of the bioassays. Eggs and fry were incubated in the dilution water used for the bioassays (Table 1). After hatching, the sac-fry were transferred to aquaria. After reaching the swim-up stage, feeding began with Rangens (Buhl, ID) trout chow starter. When sufficient fish for each bioassay had reached the feeding-fry stage, that bioassay was begun. Feeding fry were selected for testing because that brook trout life stage is considered most vulnerable to exposure to acidic water in lakes and streams (Gunn 1986). Additional eggs from the same parental source were also kept in reserve at Brandon Enterprises, where they were incubated in low calcium (2 mg/L), pH 6.5 water. Subsequently, some of these eggs at the eyed-stage were sent to the University of Wyoming in early January, 1986, for use in the bioassays.

The duration of each bioassay was 21 d. This test duration was selected because (1) this was the test length used by Ingersoll (1986) and Ingersoll et al. (1990) for brook trout feeding fry and we wanted to compare our results to theirs, which were done in the same laboratory using the same exposure system; (2) in the Adirondacks, this was the approximate length of time after ice out and snowmelt during which the fish would be most susceptible to exposure to acidic waters (Carl Schofield, Cornell University, Ithaca, NY, pers. comm.); and (3) sensitivity of feeding fry to acidic waters is believed to decrease with increasing size and 21 d seemed to be a reasonable period to test the fish before they reached a size where their sensitivities would be significantly less (Baker 1981); Cleveland et al. 1986; Hunn et al. 1987). Each bioassay was begun by serially adding five fish at a time to each exposure chamber until each chamber had 25 fry. A subsample of 30 fish from each bioassay was weighed to the nearest 0.1 mg to estimate initial mean wet weights. Mean initial weights (1 SD) for the fluoride, DOC, and temperature bioassay fry were 76.8 (7.7), 46.9 (21.3), and 59.6 (18.0) mg, respectively. Survival was checked daily.

The fish were fed twice daily ad libitum, once with Rangens trout chow starter and once with live brine shrimp (*Artemia* sp.) nauplii. Exposure chambers were cleaned twice weekly.

TABLE 1. Quality of the dilution water used in the laboratory bioassays (means and standard deviations, SD, of all measurements). All concentrations are mg/L, except pH (units) and aluminum (ppb). *T* = total Al, *R* = reactive Al, and *I* = inorganic Al.

		Aluminum											
Bioassay	pH	*T*	*R*	*I*	DOC	Ca	K	Na	Cl	F	NO_3	SO_4	°C
Fluoride	6.5	3	3	4	<1	2.2	0.9	1.1	6.9	.01	.02	1.8	10.6
SD	0.1	4	4	5	<1	0.1	0.4	0.1	1.6	<.01	0.2	0.8	0.5
DOC	6.6	2	1	1	<1	2.2	1.7	1.3	5.3	.01	.03	2.4	10.3
SD	0.2	<1	<1	<1	<1	0.4	0.9	0.1	0.5	<.01	.03	0.9	0.7
Temperature	6.7	3	2	1	<1	2.2	3.2	1.2	5.9	.01	.07	2.4	11.8
SD	0.2	2	1	<1	<1	0.4	0.7	0.7	1.0	<.01	.04	1.8	1.2

TABLE 2. Combinations (nominal concentrations) of water quality parameters tested in each of the three laboratory bioassays.

All Bioassays (*X* = experimental treatments)
2.2 ppm Ca, 12°C

		Aluminum (ppb)			
		0	111	333	1000
pH (units)	4.4	X	X	X	X
	4.8	X	X	X	X
	5.2	X	X	X	X
	5.6	X	X	X	X
	6.5	X			

Fluoride bioassay
F (ppb) added to each pH × aluminum treatment: 300, 100, 33, 0.
Dissolved organic carbon bioassay
DOC (ppm) added to each pH × aluminum treatment: 12, 6, 3, 0.
Temperature bioassay
°C for each pH × aluminum treatment: 12, 8, 4.

Experimental Design

The range of concentrations of water quality parameters tested in each bioassay (Table 2) was designed to simulate concentrations of these same water quality parameters in acidic and circumneutral lakes in the Big Moose Region of Adirondack Park, NY (Driscoll et al. 1987). These lakes are almost all present or former brook trout lakes and some are considered to have lost brook trout populations from acidification (Schofield and Driscoll 1987). All treatments were duplicated. Aluminum was not an experimental variable at pH 6.5.

Water Delivery System

A detailed description of the water delivery system is provided by Ingersoll (1986). Each combination of chemicals used in each bioassay was delivered simultaneously to the exposure chambers in a flow-through system. pH-adjusted water was delivered to four separate proportional dilution systems (modified after Mount and Brungs 1967).

For the fluoride and DOC bioassays, Marriot bottles metered either fluoride (as NaF) or DOC (as Aldrich Chemical Company humic acid, acidified to pH 5.3 with sulfuric acid) stock solutions into the pH-adjusted water at each diluter. Aluminum stock solution was added (as $A1C1_3 \times 6H_20$) via a toxicant cell with each cycling of the diluter. Each combination of chemicals was split and delivered to duplicate 600 mL exposure chambers that received 300 mL per cycle and 52 volume additions per day. At pH 6.5, aluminum was not added. However, additional fluoride and DOC controls were added with a single proportional diluter.

Water temperature was maintained at 10–12°C for all tests and controls, except for the 5° and 8°C treatments in the temperature bioassay. For the temperature bioassay, the pH-controlled water was cooled in a water bath to the desired temperature before delivery to each diluter, using plastic tubing as a heat exchanger.

Analytical Chemistry

Except for the following, analytical chemistry methods are described in Table 3.

Aluminum

The LaZerte (1984) method was ued to fractionate aqueous aluminum samples into three operationally defined fractions: total aluminum (tA1), reactive aluminum, and inorganic monomeric aluminum (imA1). The total aluminum fraction includes all dissolved and particulate aluminum species, inorganic and organic, monomeric and polymeric. The reactive aluminum fraction includes the organic monomeric and inorganic monomeric species. The inorganic monomeric fraction is that which passes through a 1000 molecular weight cutoff dialysis tubing. It primarily includes inorganic monomeric species, but may also include some low molecular weight organic species.

TABLE 3. Parameters measured and equipment and methods used for water quality measurements.

Parameter	Equipment	Method
Total Al	Perkin Elmer graphite furnace AAS model 2380	Driscoll (1984)
Reactive and inorganic Al	Perkin Elmer graphite furnace AAS model 2380	LaZerte (1984)
Ca, K, Mg, Na	Perkin Elmer flame AAS model 2380	U.S. EPA (1979)
Cl, F, NO_3, SO_4	Dionex ion chromatograph model 2110i	U.S. EPA (1979)
Organic carbon	Oceanographic International carbon analyzer, IR detector	U.S. EPA (1979)
Dissolved oxygen	YSI oxygen meter model 54A	U.S. EPA (1979)
Conductivity	Extech model 440 meter with (1 K) polymer bodied cell	U.S. EPA (1979)
Temperature	Thermometer	U.S. EPA (1979)
Humic acid DOC	Beckman Spec 20	Absorbance at 400λ
pH	Beckman model 21 meter with auto temperature compensation and glass-bodied combination pH electrode	U.S. EPA (1979)

TABLE 4. Means (standard deviations) of daily pH measurements (units) for each pH controller.

	Nominal pH				
Bioassay	4.4	4.8	5.2	5.6	6.5
Fluoride	4.40 (.07)	4.76 (.06)	5.16 (.07)	5.57 (.09)	6.45 (.13)
DOC	4.38 (.07)	4.76 (.06)	5.11 (.10)	5.56 (.10)	6.55 (.15)
Temperature	4.45 (.09)	4.78 (.06)	5.24 (.07)	5.60 (.10)	6.71 (.15)

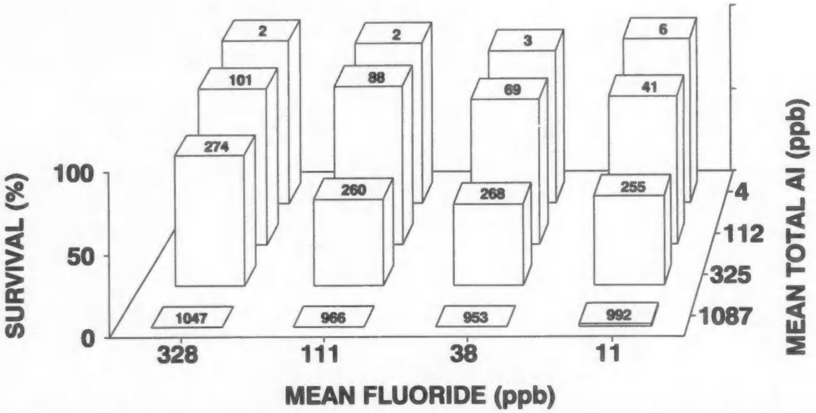

FIG. 1. Percent 21-d survival of brook trout feeding fry in the fluoride bioassay as a function of fluoride and total aluminum concentrations for all pH treatments combined. The inorganic monomeric aluminum concentration for each fluoride and total aluminum treatment is at the top of each survival bar. All values are means.

TABLE 5. Coefficients of multiple determination (R^2) and percent correct classifications (%CC) for logistic regression models from the results of the fluoride, DOC, and temperature bioassays.

Model	R^2	%CC
Fluoride bioassay		
log[F], log[tAl], pH	0.62	86
log[F], log[imAl], pH	0.64	86
log[F], log[imAl]	0.60	86
DOC bioassay		
log[DOC], log[tAl], pH	0.62	81
log[DOC], log[imAl], pH	0.67	80
log[imAl], pH	0.66	80
Temperature		
log[temperature], log[tAl], pH	0.75	83
log[temperature], log[imAl], pH	0.75	83
log[imAl], pH	0.74	83

Dissolved organic carbon

For all three bioassays, background levels of dissolved organic carbon were measured in 0.45 micron filtered water samples using total carbon analysis. For the DOC bioassay, concentrations of humic acid and DOC in 0.45 micron filtered samples representative of the range of test concentrations were measured by absorbance spectrophotometry and total carbon analysis, respectively. Then, a standard curve of absorbance vs. DOC was used to estimate DOC in additional samples.

pH

The pH of the pH-controlled water flowing to each diluter and water temperature was measured daily and adjusted, if necessary, to maintain desired pH levels (Table 4). Diluter function and the delivery of chemicals to each diluter were checked daily and adjusted, if necessary.

Sampling frequency

Except for treatments receiving no aluminum, each replicated treatment was sampled once weekly for each water quality parameter. Because background aluminum concentrations in the dilution water were vey low (3–4 ppb), treatments without additions of aluminum were sampled once during each test.

Statistics

All statistical analyses were performed using BMDP software on the University of Wyoming's Cyber 760 computer (Dixon et al. 1985; Control Data Corp.). Survival data were analyzed using stepwise logistic regression with $\log_{10}$ transformations of water quality data. Logarithmic transformations of chemical concentrations were used because they were more linearly related to logit survival than non-transformed data. The proportion of fish surviving to 21 d was analyzed for each bioassay. A coefficient of multiple determination (R^2) was estimated for each regression model.

Scatter plots and correlations between parameters were initially used to screen each independent variable and to check for multicollinearity. Independent variables were included in each final regression model if they met the following criteria: (1) the addition of the variable produced a significant ($p<0.05$) improvement in the goodness of fit chi-square; (2) the variable increased the percent correct classification of observed vs. predicted live and dead fish (Dixon et al. 1985); (3) if the signs of the variable coefficients were toxicologically reasonable (e.g.

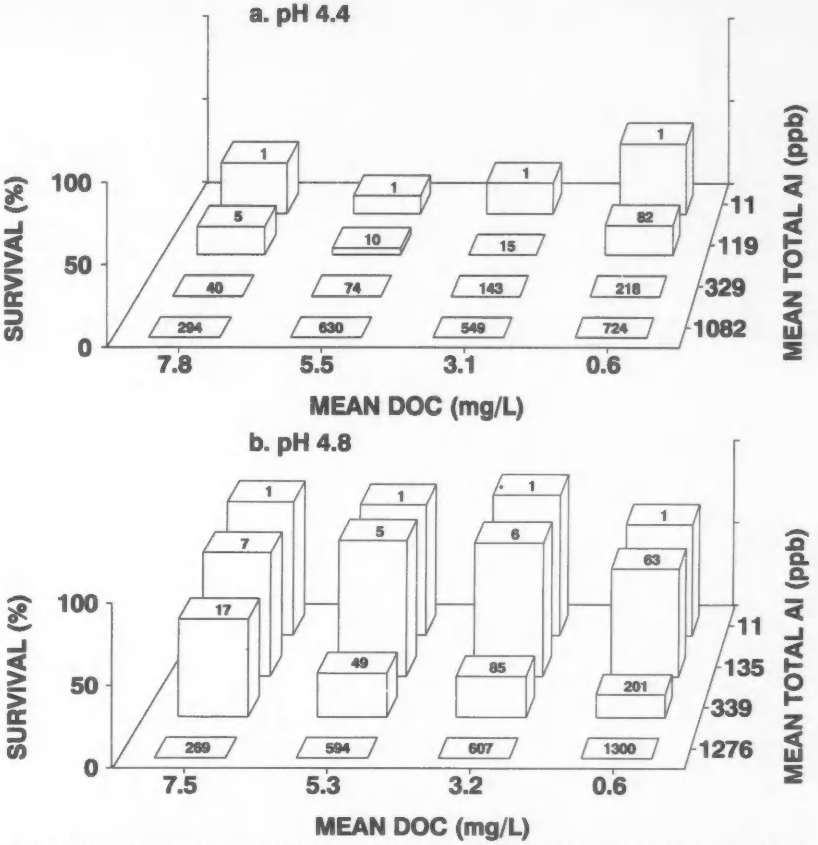

FIG. 2. Percent 21-d survival of brook trout feeding fry in the DOC bioassay as a function of DOC and total aluminum concentrations. The inorganic monomeric aluminum concentration for each DOC and

negative for inorganic monomeric aluminum and positive for pH); and (4) if the variable was not collinear with another variable. Based on these criteria, the final models selected were those that best described the data with the fewest variables.

Data Presentation

The survival data for each bioassay were summarized as three-dimensional bar graphs using Lotus 1-2-3 software (Lotus Development Corp., Cambridge, MA). The plots included only those variables found to be significant in the stepwise logistic regression analysis. Each plot summarized survival for each experimental treatment in relation to experimental water quality variables for that treatment, which included inorganic monomeric aluminum, total aluminum, and pH, plus either fluoride, DOC or temperature. All survival and water quality values are means of measured values.

The purpose of these plots is to describe overall relationships among variables. Since there were only duplicate exposure chambers per treatment, variances for each treatment can not be estimated; therefore, these plots should not be used to discern whether individual treatments are significantly different from one another. However, they can be used to illustrate significant relationships among variables identified from the results of the logistic regression analyses.

Results

Analytical Chemistry

A detailed description of the analytical chemistry results is provided by Parkhurst (1987). Tables 1 and 4 describe the water quality for each bioassay prior to the addition of aluminum, fluoride, or DOC. Total aluminum concentrations in the $4 \times 4 \times 4$ experimental matrices ranged from 3–1276 ppb, pH from 4.4–6.7, fluoride from <10–328 ppb, DOC from <1–9.2 ppm, and temperatures from 5.4–12.0°. For all parameters except pH, nominal and measured concentrations of experimental variables were not substantially different; aluminum additions tended to depress pH levels below nominal levels. Except as noted, results are discussed in terms of mean measured chemical and physical parameters for each treatment, not nominal levels.

Among the three aluminum fractions analyzed in each bioassay (total, reactive, and inorganic monomeric), concentrations of total and reactive aluminum were not significantly different ($p<0.5$) among all treatments.

Control Survival

Mean control survival (all treatments at nominal pH 6.5) was 98.9% for the fluoride bioassay, 90.5% for the DOC bioassay,

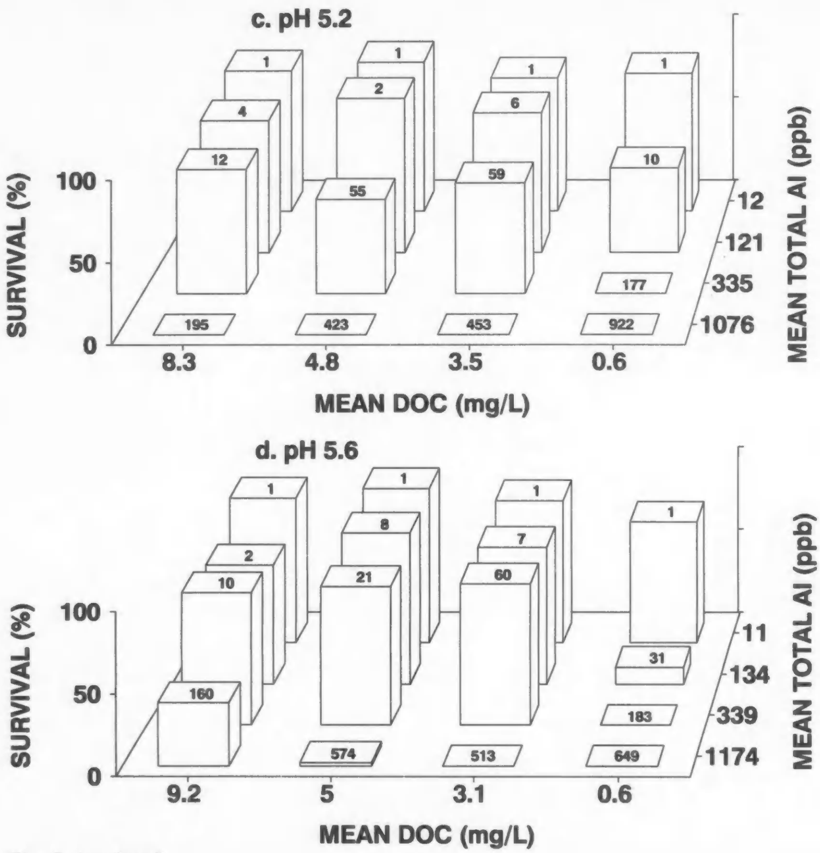

FIG. 2. *(continued)*
total aluminum treatment is at the top of each survival bar. All values are means. a, b, c, and d, increasing nominal pH.

and 87.5% for the temperature bioassay. Fluoride control survival was significantly greater ($p<0.05$) than for the DOC and temperature bioassay controls.

Fluoride Bioassay

The contribution of pH in the fluoride bioassay to the variation in survival was insignificant ($p=0.05$); therefore, pH was not included in the final logistic regression model selected for the results of the fluoride bioassay. The effect of pH was insignificant, and therefore the results of all pH treatments were combined in Fig. 1. Fluoride accounted for <2% of the variation in survival, which was still significant.

The highest total aluminum treatment (1087 ppb) caused complete mortality at all fluoride concentrations, except 11 ppb F, where 7% of the fish survived. At the two lowest aluminum concentrations (4 and 112 ppb total aluminum), none of the fluoride treatments increased survival. For treatments with 325 ppb A1, only at 328 ppb F, the highest fluoride concentration, was there a significant effect of fluoride on survival. Mean survival at this treatment was 79%, compared to a mean of 50% at all other fluoride concentrations. Thus, fluoride appears only to reduce aluminum toxicity when present at a relatively high equivalent basis; the concentration of fluoride in the treatment with 325 ppb A1 and 328 ppb F was equal to about 48% of the aluminum in equivalents per litre.

The addition of fluoride to these solutions did not reduce the concentration of inorganic monomeric aluminum, and, in fact, inorganic monomeric aluminum concentrations increased with increasing fluoride concentration, especially at the highest fluoride concentration. This indicates that the fluoride–aluminum complexes were being measured as inorganic monomeric aluminum.

Inorganic monomeric aluminum accounted for >98% of the variation in survival. Substituting total aluminum for inorganic monomeric aluminum reduced the model R^2 (Table 5), and adding interactions between variables did not improve the model R^2. Therefore, the best logistic regression model is considered only to include inorganic monomeric aluminum and fluoride:

$$\%\ \text{Survival} = e^x/(1 + e^x) \times 100, \text{ where}$$
$$x = \text{logit(survival)} = 4.26 + \log \text{F(ppb)} - 2.75 \log \text{imA1(ppb)}$$

This model has an R^2 of 0.64.

Dissolved Organic Carbon Bioassay

DOC accounted for about 6% of the variation in survival in the dissolved organic carbon bioassays, compared to 78% for

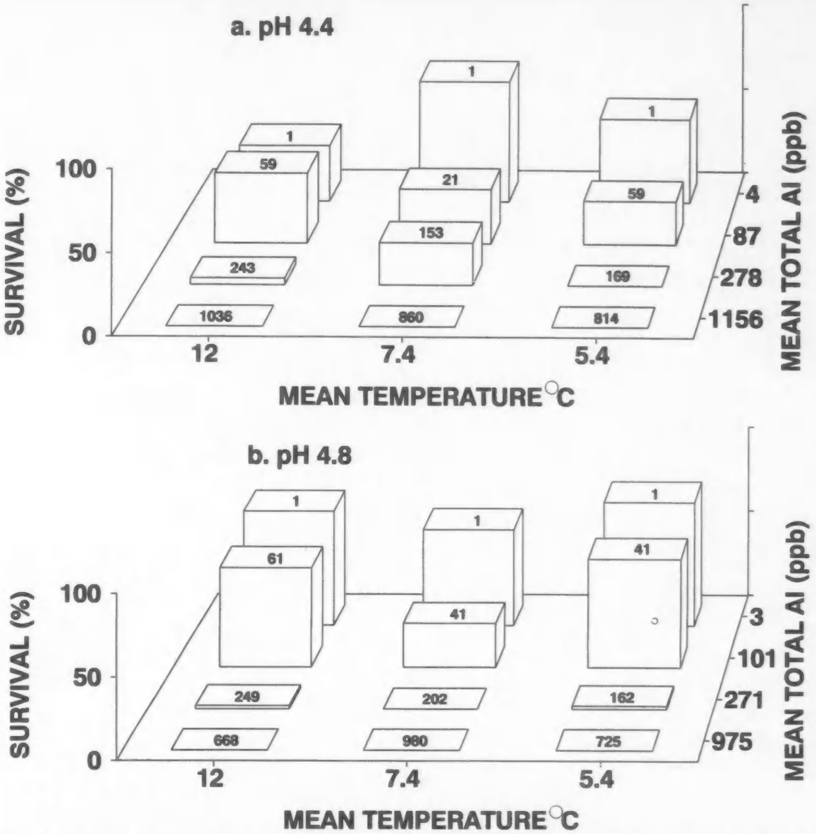

FIG. 3. Percent 21-d survival of brook trout feeding fry in the temperature bioassay as a function of temperature and total aluminum concentrations. The inorganic monomeric aluminum concentration for

inorganic monomeric aluminum and 16% for pH (Fig. 2). Increases in DOC reduced the concentration of inorganic monomeric aluminum, an effect that increased as pH increased. The effect of DOC on the concentration of inorganic monomeric aluminum was reflected in effects on survival; survival increased as both pH and DOC increased.

At low pH (Fig. 2a), the addition of DOC had the smallest effect in reducing inorganic monomeric aluminum concentrations and the smallest effect in reducing aluminum toxicity. All treatments at the two highest total aluminum concentrations had 0% survival. Treatments at pH 4.4 without added DOC (i.e. DOC <0.6 mg/L) had higher survival rates than those with DOC additions, suggesting an increase in toxicity for the organic–aluminum complexes at pH 4.4; at all higher pHs, DOC additions decreased aluminum toxicity and survival was increased relative to treatments without DOC additions.

At intermediate pHs (Fig. 2b, 2c; nominal pHs 4.8 and 5.2), the highest total aluminum treatments (i.e. ≥ 1000 ppb) still caused 100% mortality at all DOC concentrations, but DOC additions reduced total aluminum toxicity at all lower total aluminum concentrations. At the highest pH treatment (Fig. 2d, nominal pH 5.6), the effects of DOC in reducing aluminum toxicity were most dramatic. The highest DOC treatments, 5 and 9.2 mg/L, even reduced aluminum toxicity at the highest total aluminum concentration, 1174 ppb; 21-d survival was 2 and 38%, respectively.

Substituting total aluminum for inorganic monomeric aluminum in the logistic regression analysis reduced the model R^2, and adding interactions between variables did not improve the model R^2; therefore, the best logistic model is considered to include DOC, inorganic monomeric aluminum and pH;

$$\text{logit survival} = -9.34 + 1.99\ \text{pH} + 0.62\ \log[\text{DOC}] - 0.90\ \log[\text{-imAl}]$$

This model had an R^2 of 0.67 (Table 5).

Temperature Bioassay

In the temperature bioassay, the toxicities of aluminum and pH overwhelmed the effects of temperature on survival. Temperature had a small but statistically significant effect on survival, accounting for <1% of the explained variation in survival, compared to about 3% for pH and 96% for inorganic monomeric aluminum. Increases in temperature tended to decrease survival, but, overall, temperature effects were small and inconsistent (Fig. 3).

Either total or inorganic monomeric aluminum combined with pH accounted for virtually all of the explained variation in survival. The models with inorganic monomeric aluminum and total aluminum had R^2 values of 0.75 (Table 5). The best models were considered to be either

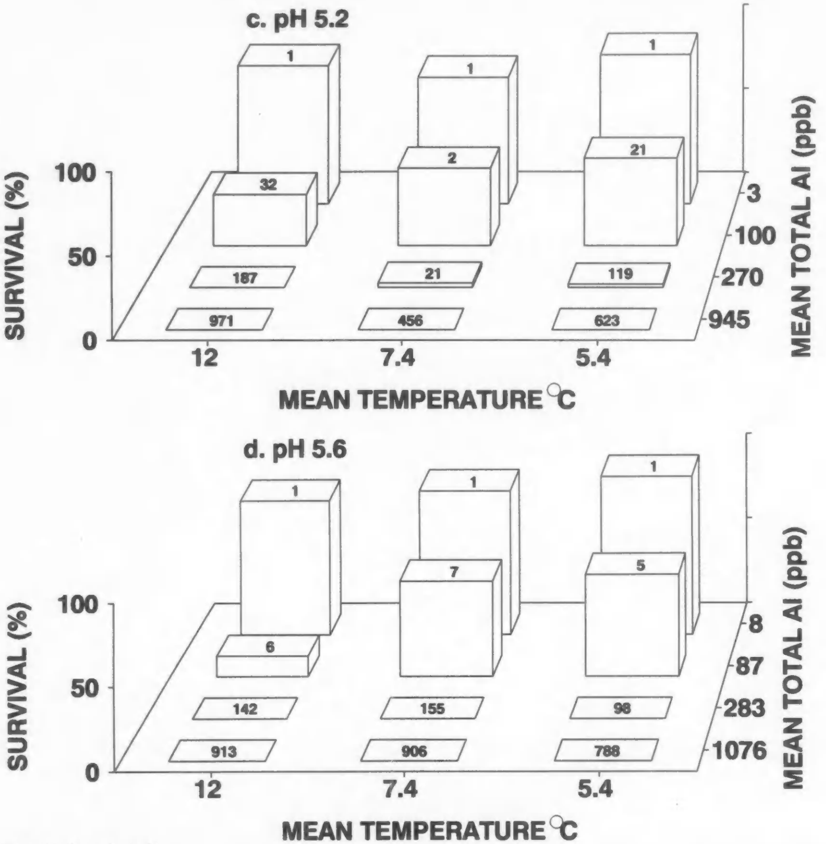

FIG. 3. *(continued)*
each temperature and total aluminum treatment is at the top of each survival bar. All values are means. a, b, c, and d, increasing nominal pH.

$$\text{logit survival} = -1.99 + 0.76\ \text{pH} - 1.19 \log[\text{temperature}] - 1.40 \log[\text{imAl}]$$

or

$$\text{logit survial} = -1.65 + 0.93\ \text{pH} - 1.51 \log[\text{temperature}] - 1.53 \log[\text{tAl}].$$

Discussion

Among a wide range of fluoride, DOC and temperature values, the concentration of inorganic monomeric aluminum was the primary determinant of brook trout feeding fry survival in acidic aluminum solutions. Inorganic monomeric aluminum had a much greater relative effect on survival of brook trout feeding fry than pH, which was also the conclusion of Ingersoll (1986), Baker (1981), and Johnson et. al. (1987). Except in the fluoride bioassay, where pH was found to be an insignificant variable, pH was the second-most important variable, accounting for up to 16% of the variation in survival.

Fluoride, DOC, and temperature all had small, but significant contributions to survival. Fluoride or temperature each accounted for <2% of the variation in survival, while DOC accounted for 6% of the variation in survival.

Among all treatments in all three bioassays in which DOC levels were <1 ppm, (i.e. all treatments not receiving DOC additions) total, reactive, or inorganic monomeric aluminum were equivalent estimators of brook trout survival in acidic waters. Because the addition of DOC complexed total aluminum, significantly reducing concentrations of inorganic monomeric aluminum in test waters with DOC levels >1 ppm, (i.e. all treatments with DOC additions) the inorganic monomeric aluminum fraction was a better predictor of the toxicity of aluminum than total aluminum or reactive aluminum.

To further illustrate this relationship, total and inorganic aluminum concentrations alone vs. percent survival in all treatments of the DOC bioassays (i.e. pH 4.4–5.6) were analyzed using simple least-squares regression. Since survival data are binomially distributed, a log-odds model was used, which is a linear form of the logistic regression model (Montgomery and Peck 1982). As in the logistic regression analyses, the aluminum data were converted to $\log_{10}$ of the concentration (ppb). The linear regression analysis showed that, while both total and inorganic monomeric aluminum were significantly correlated with survival, the R^2 value for the regression analysis with inorganic monomeric aluminum (0.68) was substantially larger than the R^2 value for the regression analysis with total aluminum (0.39). Thus, inorganic monomeric aluminum explained a much higher proportion of the variation in survival than did total aluminum.

Since most surface waters have DOC concentrations >1 ppm, the concentration of inorganic monomeric aluminum should provide the best prediction of aluminum toxicity over the widest range of water quality conditions. These results support the conclusions of Baker (1981) and Driscoll et al (1980).

Since DOC, fluoride, and/or temperature explain an additional 1–6% of the variation in survival, it may seem desireable to include them as variables in logistic regression models for estimating brook trout survival in acidic waters. However, Parkhurst (1987) reported that all of these variables were significantly correlated with inorganic monomeric aluminum concentrations and pH at nine lakes and streams in the Adirondack Mountains, New York, which were used to evaluate the in situ toxicity of acidic waters to brook trout feeding fry. Therefore, including additional, correlated variables in logistic regression models would not increase the abilities of the models to estimate survival, and models that include just pH and inorganic monomeric aluminum should be adequate for estimating in situ survival of brook trout feeding fry.

The results of these bioassays support the hypothesis tested in this study. That is, over a wide range of pH and aluminum concentrations, together with a wide range of fluoride and DOC concentrations and water temperatures, pH and the concentration of inorganic monomeric aluminum are the best predictors of the toxicity of acidic waters to brook trout fry. These results generally support the conclusions of Baker and Schofield (1982), who also found that inorganic monomeric aluminum was the primary factor affecting brook trout survival in acidic waters.

References

Baker, J. P. 1981. Aluminum toxicity to fish as related to acid precipitation and Adirondack surface water quality, PhD dissertation, Cornell University, Ithaca, NY. 441 p.

1983. Fishes, p. 5–76 to 5–137. *In* The acidic deposition phenomenon and its effects. Critical assessment review papers, Vol. II, Effects Sciences. Public Review Draft, EPA-600/8-83-0168. U.S. Environmental Protection Agency, Washington, D.C.

Baker, J. P., and C. L. Schofield. 1982. Aluminum toxicity to fish in acidic water. Water Air Soil Pollut. 18: 289–306.

Bergman, H. L., J. S. Mattice, and D. J. A. Brown. 1988. Lake acidification and fisheries project: adult brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1563–1574.

Brown, D. J. A. 1981 The effects of various cations on the survival of brown trout, *Salmo trutta* at low pHs. J. Fish. Biol. 18: 31–40.

Cleveland, L., E. E. Little, S. J. Hamilton, D. R. Buckler, and J. B. Hunn. 1986. Interactive toxicity of aluminum and acidity to early life stages of brook trout. Trans. Am. Fish. Soc. 115: 610–620.

Clark, K. L., and B. D. Lazerte 1985. A laboratory study of the effects of aluminum and pH on amphibian eggs and tadpoles. Can. J. Fish. Aquat Sci. 42: 1544–1551.

Dalziel, T. R. K., R. Morris, and D. J. A. Brown. 1986. The effects of low pH, low calcium concentrations and elevated aluminum concentrations on sodium fluxes in brown trout, *Salmo trutta* L. Water Air Soil Pollut. 30: 569–577.

Dixon, W. J., M. B. Brown, L. Engelman, J. W. Frane, M. A. Hill, R. I. Jennrich, and J. D. Toporek. 1985. BMDP statistical software. University of California Press, Berkeley, CA. 734 p.

Drever, J. I. 1982. The geochemistry of natural waters. Prentice–Hall Inc., Englewood Cliffs, NJ. 388 p.

Driscoll, C. T. 1983. Aluminum chemistry in acidic waters, p. 4–99 to 4–108. *In* The acidic deposition phenomenon and its effects. Critical assessment review papers, Vol. II, Effects Sciences. Public Review Draft, EPA-600/8-83-0168. U.S. Environmental Protection Agency, Washington, D.C.

1984. A procedure for the fractionation of aqueous aluminum in dilute acidic waters. Int. J. Environ. Anal. Chem. 16: 267–283.

Driscoll, C. T., Jr., J. P. Baker, J. J. Bisogni, Jr., and C. L. Schofield. 1980. Effect of aluminum speciation on fish in dilute acidified waters. Nature (Lond.) 284: 161–164.

Driscoll, C. T., C. P. Yatsko, and F. J. Unangst. 1987. Longitudinal and temporal trends in the water chemistry of the North Branch of the Moose River. Biogeochemistry 3: 37–62.

Fivelstad, S., and H. Leivestad. 1984. Aluminum toxicity to Atlantic salmon *Salmo salar* L. and brown trout *Salmo trutta* L.: Mortality and physiological response. Inst. Freshwater Res. Drottningholm 61: 69–77.

Gunn, J. M. 1986. Behaviour and ecology of salmonid fishes exposed to episodic pH depressions. Environ. Biol. Fishes 17: 241–252.

Gunn, J. M., and D. L. G. Noakes. 1986. Avoidance of low pH and elevated A1 concentrations by brook charr *Salvelinus fontinalis* alevins in laboratory tests. Water Air Soil Pollut. 30: 497–503.

Hunn, J. B., L. Cleveland, and E. E. Little. 1987. Influence of pH and aluminum on developing brook trout in a low calcium water. Environ. Pollut. 43: 63–73.

Hutchinson, N. J., and K. E. Holtze. 1985. Biological effects of acidification. XI. Factors influencing the acute lethality of hydrogen and A1 ion to lake and brook trout, p. 104. *In* The International Symposium on Acid Precipitation. Muskoka Conference 1985. Muskoka, Ont. 428 p.

Ingersoll, C. G. 1986. The effects of pH, aluminum, and calcium on survival and growth of brook trout (*Salvelinus fontinalis*) early life stages. PhD. dissertation, University of Wyoming, Laramie, WY. 122 p.

Ingersoll C. G., D. R. Mount, D. D. Gulley, T. W. LaPoint, and H. L. Bergman. 1990. Effects of pH, aluminum, and calcium on survival and growth of eggs and fry of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 000–000.

Johnson, D. W., H. A. Simonin, J. R. Colquohoun, and F. M. Flack. 1987. In situ toxicity tests of fishes in acid waters. Biogeochemistry 3: 191–208.

LaZerte, B. D. 1984. Forms of aqueous aluminum in acidified catchments of central Ontario: a methodological analysis. Can. J. Fish. Aquat. Sci. 41: 766–776.

Martin, H. C. 1987. Acidic precipitation. 1987. D. Reidel Publishing Co., Dordrecht, Holland, 1118 p.

Montgomery, D. C., and E. A. Peck. 1982. Introduction to linear regression analysis. John Wiley and Sons, New York, NY. 504 p.

Mount, D. I., and W. A. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. Water Res. 1: 21–29.

Mount, D. R., C. G. Ingersoll, D. D. Gulley, J. D. Fernandez, T. W. LaPoint, and H. L. Bergman. 1988. Effect of long-term exposure to acid, aluminum, and low calcium on adult brook trout *Salvelinus fontinalis*. 1. Survival, growth, fecundity, and progeny survival. Can. J. Fish Aquat. Sci. 45: 1623–1632.

Mount, D. R., M. J. Swanson, J. E. Breck, A. M. Farag and H. L. Bergman. 1990. Responses of brook trout *Salvelinus fontinalis* fry to fluctuating acid, aluminum, and low calcium exposure. Can. J. Fish. Aquat. Sci. 47: 1623–1630.

Muniz, I. P., and H. Leivestad. 1980. Toxic effects of aluminum on the brown trout, *Salmo trutta* L., p. 320–321. *In* D. Drablos and A. Tollan [ed.] Proceedings of an international conference on the ecological impact of acid precipitation. SNSF Project: effects on forest and fish. Oslo, Norway.

Parkhurst, B. R. 1987. A comparison of laboratory and in situ bioassays for evaluating the toxicity of acidic waters to brook trout. PhD. dissertation, University of Wyoming, Laramie, WY. 131 p.

Rosseland, B. O., and O. L. Skogheim. 1984. A comparative study on salmonid fish species in acid aluminum-rich water. II. Physiological stress and mortality of one- and two-year-old fish. Inst. Freshwater Res. Drottningholm 61: 186–194.

Schofield, C. L., and J. R. Trojnar. 1980. Aluminum toxicity to brook trout *Salvelinus fontinalis* in acidified waters. *In* T. Y. Toribara, M. W. Miller, and P. E. Morrow [ed.] Polluted Rain. Plenum Press, New York, NY. p. 341–362.

Schofield, C. L., and C. T. Driscoll. 1987. Fish species distribution in relation to water quality gradients in the North Branch of the Moose River Basin. Biogeochemistry 3: 63–86.

Siddens, L., W. K. Seim, L. R. Curtis, and G. A. Chapman. 1986. Comparison of continuous and episodic exposure to acidic, aluminum-contaminated waters of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 43: 2036–2040.

Siep, H. M., L. Muller, and A. Naas. 1984. Aluminum speciation: comparison of two spectrophotometric analytical methods and observed concentrations in some acidic aquatic systems in southern Norway. Water Air Soil Pollut. 23: 81–95.

SKOGHEIM, O. K., AND B. O. ROSSELAND. 1984. A comparative study on salmonid fish species in acid aluminum-rich water. I. Mortality of eggs and alevins. Inst. Freshwater Res. Drottningholm 61: 177–185.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY (U.S. EPA). 1979. Methods for chemical analysis of water and wastes. EPA-600/4-79-020. U.S. Environmental Protection Agency, Washington, D.C. 298 p.

WOOD, C. M., D. G. MCDONALD, C. G. INGERSOLL, D. R. MOUNT, O. E. JOHANNSSON, S. LANDSBURGER, AND H. L. BERGMAN. 1990a. Effects of water acidity, calcium, and aluminum on whole body ions of brook trout (*Salvelinus fontinalis*) continuously exposed from fertilization to swim-up: a study by instrumental neutron activation analysis. Can. J. Fish. Aquat. Sci. 47: 1593–1603.

WOOD, C. M., D. G. MCDONALD, C. G. INGERSOLL, D. R. MOUNT, O. E. JOHANNSSON, S. LANDSBURGER, AND H. L. BERGMAN. 1990b. Whole body ions of brook trout (*Salvelinus fontinalis*) alevins: responses of yolk-sac and swim-up stages to water acidity, calcium, and aluminum, and recovery effects. Can. J. Fish. Aquat. Sci. 47: 1604–1615.

Aluminum and Acid Toxicity to Two Strains of Brook Trout (*Salvelinus fontinalis*)

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Ingersoll, C. G., D. D. Gulley, D. R. Mount, M. E. Mueller, J. D. Fernandez, J. R. Hockett, and H. L. Bergmann. 1990. Aluminum and acid toxicity to two strains of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 1641–1648.

Two strains of 1 yr-old brook trout were exposed to 14 combinations of pH, aluminum, and calcium during a 28-d experiment. Survival, weight, and gill histology of both strains were affected similarly by pH, aluminum, and calcium exposure. Survival was reduced at inorganic monomeric aluminum concentrations (IA1) of 29 µg/L at pH 5.2 and ≥228 µg/L at pH 4.4 and 4.8. Fish weight was reduced with exposure to pH below 4.8 or aluminum concentrations ≥34 µg/L. The gills sampled from fish exposed to low pH exhibited lifting of the outer epithelium and hypertrophy (increase in size) of chloride and epithelial cells. The response of gills sampled from fish exposed to low pH and elevated aluminum was more pronounced relative to the low pH exposure alone. In addition to the effects observed in the low pH exposed fish, the gills from fish exposed to both acid and aluminum exhibited vacuolation and degeneration of epithelial and chloride cells and the presence of dense cells. Finally, fish confined in PVC tubes designed to mimic in situ field exposure procedures were more sensitive to the toxic effects of acid and aluminum compared to free-swimming fish. Caution must be taken when extrapolating safe field levels from in situ exposures of caged fish.

Deux lignées d'omble de fontaine âgé de un an ont été exposées à 14 combinaisons de pH, de concentrations d'aluminium et de calcium au cours d'une expérience de 28 jours. L'exposition à différents pH, aux différentes concentrations d'aluminium et de calcium a modifié de la même façon la survie, le poids et la structure histologique des branchies chez les deux lignées. La survie a été réduite lorsque les concentrations de composés inorganiques monomériques à base d'aluminium étaient de 9 µg/L à pH 5,2 et supérieures ou égales à 228 µg/L à pH 4,4 à 4,8. Le poids des poissons était réduit lorsqu'ils étaient exposés à un pH inférieur à 4,8 ou à des concentrations d'aluminium supérieures ou égales à 34 µg/L. Les échantillons de branchies prélevés chez des poissons exposés à un pH faible présentaient un soulèvement de la couche extérieure de l'épithélium et une hypertrophie (augmentation de la taille) des cellules acidophiles (dites à chlorure) et des cellules épithéliales. La réaction des branchies échantillonnées provenant de poisson exposés à un pH faible et à une concentration élevée d'aluminium était plus marquée par rapport à une exposition à pH faible seulement. En plus des effets observés chez les poissons exposés à un pH faible, les branchies des poissons exposés à un milieu acide et à l'aluminium présentaient une vacuolisation et une dégénération des cellules épithéliales et des cellules acidophiles et on observait la présence de cellules denses. Finalement, des poissons placés dans des tubes de PVC conçus pour reproduire les méthodes d'exposition sur le terrain in situ étaient plus vulnérables aux effets toxiques du milieu acide et de l'aluminium que les poissons en liberté. Il faut être prudent lorsque l'on extrapole des niveaux sans danger sur le terrain à partir d'expositions in situ de poissons en milieu restreint.

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Surface waters in several regions of North America (Haines and Baker 1986) and Europe (Rosseland et al. 1986) are perceived to be threatened by acidification and fisheries in many of these locations are thought to have been reduced or lost (Baker and Schofield 1985). An understanding of the effects of acid, aluminum, and calcium on fish populations is needed (Baker and Schofield 1980; Driscoll et al. 1980; Sevaldrud and Skogheim 1986). Interspecific differences in both acid and aluminum toxicity have been extensively documented (e.g. Baker and Schofield 1982; Hulsman et al. 1983; Baker 1984; Fivelstad and Leivestad 1984; Rosseland and Skogheim 1984). While intraspecific differences in the survival to low pH have been observed (Johnson 1975; Gjedrem 1976; Robinson et al. 1976; Swarts et al. 1978; Edwards and Gjedrem 1979; Rahel 1983; Rask and Virtanen 1986; Schom 1986) intraspecific comparisons among fish strains to aluminum sensitivity have not been documented.

The objectives of the present study were to determine the effects of pH, aluminum, and calcium exposure in the laboratory on: (1) the survival, growth, and gill histology of two strains of 1 yr-old brook trout, and (2) the response of fish confined in enclosures that mimic in situ field exposure conditions. Other papers in this issue (Bergman et al. 1990) and in a previous issue of this journal (Bergman et al. 1988) present additional toxicological, physiological, and histopathological results on early life stage and adult brook trout exposed in the laboratory to water conditions typical of acidified lakes and streams.

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TABLE 1. Nominal pH levels and aluminum concentrations (μg/L). Nominal calcium concentration for each pH and aluminum combination was 2.0 mg/L, except for pH 4.4 where 1.0 mg/L was also tested.

pH	Aluminum (μg/L)			
6.5	0	—	—	—
5.2	0	111	—	—
4.8	0	111	333	—
4.4	0	111	333	666

Materials and Methods

Exposure Conditions

Two strains of 1 yr-old brook trout were exposed to 14 combinations of pH, aluminum, and calcium during a 28-d experiment (10 October–7 November 1985) (Table 1). Fish were exposed to higher aluminum concentrations at lower pH levels, because dissolved inorganic aluminum concentration increases as pH decreases in acidified surface waters (e.g. Schofield 1982). Well water (250 mg/L hardness as $CaCO_3$, pH 7.8) treated by reverse osmosis and deionization (Ingersoll 1986) was delivered to an 800-L head tank where 1 mg Na/L was added as NaCl. The pH of this water was adjusted to pH 6.5 with a pH controller (Leeds and Northrup, Model 7083) using either dilute H_2SO_4 or KOH. This pH-adjusted water was chilled and mixed by a 1-hp chiller (Frigid Units Model D1-100, Toledo, OH) and then delivered to four head tanks where the pH was lowered in three of these head tanks (again using dilute H_2SO_4 and pH controllers). Circulating pumps (Model AC-2CP-MD, March Mfg. Inc., Glenview, IL) vigorously mixed and aerated the water in these tanks. This pH-adjusted water was delivered to continuous flow serial diluters where calcium (as $CaCl_2$) and aluminum (as $AlCl_3$) were added using Mariotte bottles. Each unique combination of pH-, calcium-, and aluminum-adjusted water was delivered to 340-L Nalgene exposure tanks that received 1 L/min resulting in 4.2 volume additions daily. The pH of exposure tanks at low aluminum concentrations (0–111 μg/L) tended to increase relative to the inflowing pH adjusted water, so additional Mariotte bottles were used to deliver dilute sulfuric acid to these tanks to manually lower the pH to the desired level.

Strain Comparison

One strain of brook trout was obtained from Fernwood Trout Hatchery, Gansevoort, New York (Temiscamie × domestic strain) and a second strain was obtained from Beity's Resort, Valley, Washington (unknown origin). Both groups were held in well water at 10°C from July 1985 until 3 September 1985. Photoperiod was adjusted biweekly to simulate the annual cycle for Denver, Colorado during this time period. These fish were then acclimated to water adjusted to pH 6.5 and 2 mg Ca/L without aluminum addition. After a 38-d acclimation to this soft water, 50 fin-clipped 1 yr-old New York and Washington strain fish (e.g. 100 fish in total) were placed into each exposure tank at a 16-h light 8-h darkness photoperiod. Mean starting weights for New York- and Washingon-strain fish were 10.5 g (standard error of the mean; SEM = 0.53) and 9.3 g (SEM = 0.44), respectively. Fish were fed a commercial trout food (Rangens, Buhl, ID) at about 2% of their body weight per day and the exposure tanks were siphoned daily. Mortality was recorded daily; wet weight of fish was determined at the end of the experiment.

Histopathology

Five New York- and Washington-strain fish were sampled for gill histology after the 28-d exposure to 2 mg Ca/L and: (1) pH 6.5, 0 μg Al/L, (2) pH 4.4, 0 μg Al/L, and (3) pH 4.4, 333 μg Al/L (nominal). Each fish was sacrificed by severing the spinal cord just behind the operculum. The second gill arch on the right side was immediately excised, fixed in 2.5% glutaraldehyde (buffer: 0.08 M Sorenson's at pH 7.4, 20°C) for 24 h, and post-fixed for 2 h in 1% buffered osmium solution at 20°C. After dehydration in a series of ethanol rinses (50, 75, 95 and 100%) and propylene oxide (100%), each gill arch was embedded in Araldite-502 epoxy resin which was polymerized at 60°C for 24 h. One-micrometer-thick oblique sections of the gill were cut with a Sorvall MT2-B Porter Blum Ultramicrotome. The same sectioning plane was used on all of the samples to ensure valid structural comparisons; this plane was oriented to transect the ventral arm of the gill arch near the sharp bend formed by the intersection of the dorsal and ventral arms of the arch. This sectioning plane transects secondary lamellae on the posterior side of each filament in cross section (Johnson 1983; Tietge et al. 1988). Sections were stained for 1 min with 0.2%

TABLE 2. Mean survival and final weight (SEM) for the New York-strain (NY-c) fish confined in PVC tubes and free-swimming New York- (NY-fs) and Washington-strain (WA-fs) fish. Measured (SEM) pH, calcium (mg/L) and nominal (Al), total (TA1), monomeric (MA1) and inorganic monomeric (IA1) aluminum (μg/L) are listed.

						Survival (%)			Weight (g)		
pH	Ca	Al	TAl	MAl	IAl	WA-fs	NY-fs	NY-c	WA-fs	NY-fs	NY-c
6.52(0.05)	1.86(0.07)	0	5(1.43)	7(0.85)	4(0.96)	100.0	100.0	90.0	11.5(0.65)	13.2(0.79)	8.2(1.29)
5.24(0.03)	1.89(0.10)	0	5(0.66)	7(0.61)	3(0.42)	98.0	100.0	100.0	10.5(0.68)	15.6(0.90)	6.7(0.64)
5.16(0.03)	1.78(0.12)	111	118(23.1)	103(9.55)	29(11.3)	78.0	89.6	100.0	7.8(0.58)	10.0(0.67)	7.5(0.99)
4.77(0.03)	1.76(0.19)	0	4(0.81)	7(0.97)	4(0.60)	100.0	100.0	90.0	8.7(0.47)	13.6(0.77)	7.6(1.26)
4.79(0.03)	1.83(0.12)	111	74(18.9)	105(20.5)	42(13.2)	98.0	100.0	90.0	7.2(0.52)	12.0(0.68)	9.2(1.65)
4.68(0.02)	1.76(0.08)	333	191(84.2)	261(95.7)	237(54.7)	57.4	75.5	80.0	6.0(0.52)	8.3(0.52)	7.0(0.45)
4.44(0.02)	1.85(0.12)	0	5(0.79)	8(0.34)	4(0.30)	96.1	100.0	70.0	8.6(0.56)	10.9(0.78)	10.1(1.27)
4.40(0.02)	1.75(0.15)	111	131(47.6)	115(9.26)	71(15.8)	92.3	98.0	27.3	6.5(0.34)	8.4(0.49)	6.5(1.28)
4.38(0.01)	1.80(0.07)	333	322(70.6)	311(118.)	228(83.0)	88.0	80.4	40.0	5.9(0.37)	7.8(0.43)	8.2(0.97)
4.33(0.01)	1.85(0.08)	666	665(186.)	646(46.2)	552(81.3)	20.8	42.9	20.0	5.0(0.72)	8.9(1.03)	12.2(1.30)
4.43(0.02)	1.05(0.05)	0	8(1.67)	7(0.57)	5(1.33)	100.0	100.0	77.8	7.6(0.48)	11.6(1.01)	8.1(1.15)
4.46(0.02)	1.02(0.02)	111	108(25.0)	91(11.8)	34(11.0)	98.0	98.0	90.0	6.9(0.41)	9.4(0.50)	8.2(0.93)
4.39(0.01)	0.98(0.04)	333	296(27.1)	333(20.7)	239(41.6)	77.4	68.8	50.0	6.4(0.50)	7.9(0.67)	9.5(1.37)
4.32(0.01)	0.96(0.02)	666	704(133.)	662(35.7)	443(107.)	14.0	2.0	0.0	6.0(0.60)	—	—

azure-B (0.25 M sodium borate at pH 8.1) and observed using a Zeiss universal transmitted-light microscope employing Nomárski differential interference contrast and Kolhler illumination. Each gill section was examined for qualitative changes in the secondary lamellae including: (1) general pathology, (2) distribution of pillar, chloride, and dense cells, and (3) changes in diffusion distance and lymphatic spaces.

Enclosure Comparison

PVC tubes (6-inch diameter pipe, 45-cm long) were used to confine 10 New York-strain fish in each exposure tank. Nylon-netting (1/4-inch mesh) was secured to both ends of each tube to restrain these fish and keep them from feeding. The purpose of these enclosures was to mimic in situ exposure methods that are often used in field caging studies with fish (e.g. Kennedy 1980; Johnson et al. 1987; Parkhurst et al. 1990).

Water Chemistry

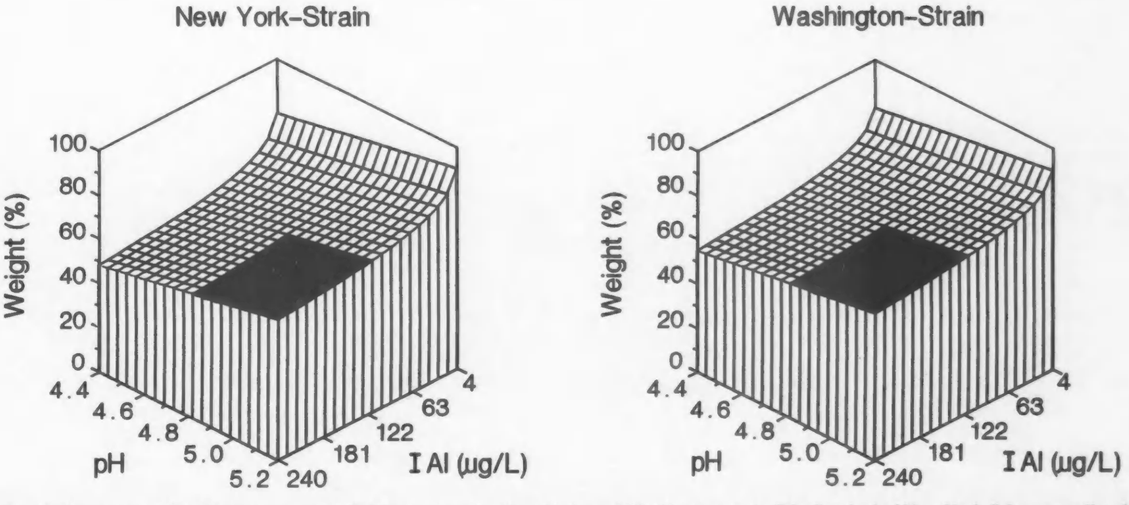
The flow rate of calcium and aluminum stock solution from each Mariotte bottle was measured daily and adjusted when necessary. Exposure tank pH (Orion Ross combination pH electrode), temperature, and conductivity were measured daily. Cations (Ca, Na, Al) and anions (Cl, SO_4, F) were measured weekly with a Perkin-Elmer (Model 2380) atomic absorption spectrophotometer and a Dionex (Model 2110i and a #AS-40 column) automated single-channel ion chromatograph, respectively. The LaZerte (1984) method was used to fractionate aqueous aluminum into three operationally defined fractions: total aluminum (TA1), total monomeric (fast reactive) aluminum (MA1), and inorganic monomeric (dialysis/fast reactive) aluminum (IA1). The total aluminum fraction includes all dissolved and particulate aluminum species, (monomeric and polymeric, inorganic and organic). The total monomeric aluminum fraction mostly includes the organic monomeric and inorganic monomeric species. The inorganic monomeric aluminum fraction primarily consists of inorganic aluminum species, but might also include some small organic species.

Measured pH and calcium levels were in close agreement with nominal values (Table 2). While total and monomeric aluminum averaged about 93% of nominal, the inorganic monomeric aluminum concentrations averaged about 65% (range 24–124%) of both the measured total and monomeric aluminum concentration (Table 2). In Ingersoll et al. (1990) we report the majority of inorganic monomeric aluminum to be monomeric aluminum (e.g. little organic complexation). In that early life stage brook trout study, the fish loading was about 275 mg/L/h, whereas in the present study with juveniles, the fish loading was about 18 g/L/h. While exposure water dissolved organic carbon (DOC) concentrations were not measured in either study, the higher fish loading rate in the present study probably resulted in elevated organic complexation of aluminum. In addition, inorganic monomeric aluminum was higher in proportion to total aluminum in the exposure chambers where fish loading was lowest because of mortality (≥ 333 μg Al/L and pH ≤ 4.8; Table 2). Regression models for survival and growth data based on inorganic monomeric aluminum (Fig. 1 and 2) explained the most variance with the fewest variables compared to models based on nominal, total, or monomeric aluminum. This is in agreement with Driscoll et al. (1980) and Parkhurst (1987) who found inorganic monomeric aluminum to be the fraction most toxic to fish.

Average measured cation and anion concentrations of the exposure water in mg/L were: sodium 1.1 (0.01 SEM, range 1.0–1.1), potassium 4.70 (0.02 SEM, range 4.6–4.9), magnesium 0.02 (0.003 SEM, range 0.01–0.02), fluoride 0.015 (0.001 SEM, range 0.009–0.024), chloride 4.91 (0.25 SEM, range 3.2–7.0), and sulfate 8.70 (0.67 SEM, range 0–10.3). Dissolved oxygen was greater than 76% saturation (range 6.2–8.5 mg/L) and temperature averaged 11.6°C (0.08 SEM, range 11.3–12.1).

Statistics

Survival data were analyzed with stepwise logistic regression. Proportion surviving was assumed to fit a logistic distribution:


New York–Strain

Washington–Strain

FIG. 1. Response surface for percent survival of free-swimming New York- and Washington-strain fish. Survival of New York fish was predicted by the equation: Logit survival = -1.90 pH $-$ 3.46 log(IAl) + 17.8 ($R^2=0.87$). Survival of Washington fish was predicted by the equation: Logit survival = -3.01 pH $-$ 2.55 log(IAl) + 20.7 ($R^2=0.81$). The response predicted in the darkened area should not be interpreted since these combinations of pH and aluminum were not tested.

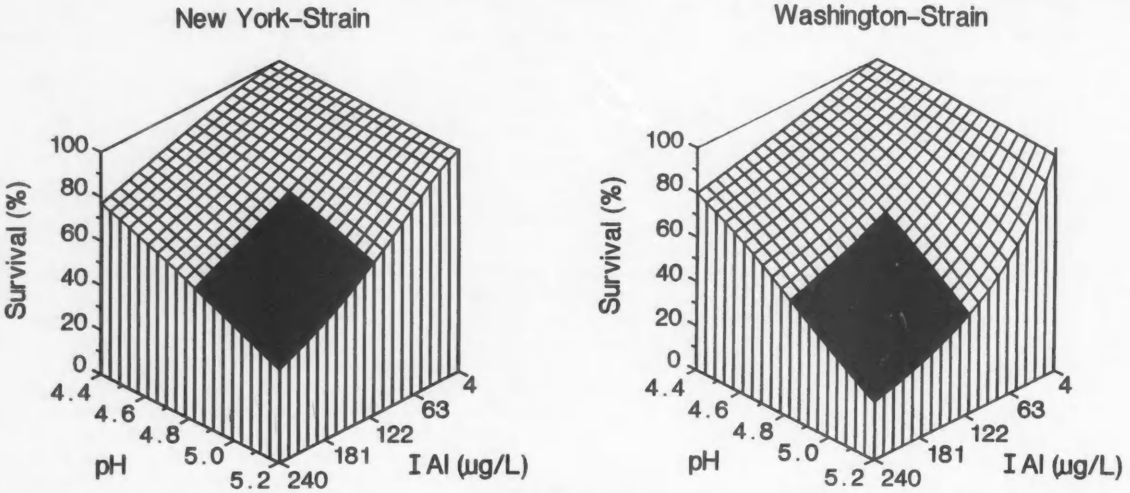


FIG. 2. Response surface for ending weight (relative to pH 5.2 without aluminum addition) of free-swimming New York- and Washington-strain fish. Weight of New York fish was predicted by the equation: Weight (g) = 3.02 pH − 2.38 log(IAl) − 1.14 × 10^{-1} ($R^2 = 0.93$). Weight of the Washington fish was predicted by the equation: Weight (g) = 1.59 pH − 1.38 log(IAl) + 2.08 ($R^2 = 0.96$). The response predicted in the darkened area should not be interpreted since these combinations of pH and aluminum were not tested.

$$E(y) = \frac{\exp(\beta_0 + \beta_1 X_1 \ldots + \beta_n X_n)}{1 + \exp(\beta_0 + \beta_1 X_1 \ldots + \beta_n X_n}$$

where y is the proportion surviving, β_0 to β_n are the parameters of the regression model, and X_1 to X_n are the independent variables. Weight data were analyzed with linear stepwise regression to predict the effects of pH, calcium, and aluminum. All dependent variables were regressed against both non-transformed and $\log_{10}$-transformed chemistry values, except for acidity which was always expressed as pH. The purpose of the regression analyses was to define simple models that would identify the key variables relating to each measured response. For logistic regressions, variables were included if they significantly improved (alpha = 0.05) the goodness of fit Chi-square. For linear regressions, the F statistic was used to determine the significance of the regression (alpha = 0.05), and only variables that increased the adjusted R^2 of the model were considered for inclusion in the final model (Mount et al. 1988; Ingersoll et al. 1990).

Three-dimensional response surfaces were plotted using the regression models and Golden Graphics Software (Golden, CO). At pH 6.5, aluminum was not an experimental variable, so these data were not included in either the regression models or the response surfaces. Aluminum was not tested above 111 and 333 μg/L at pH 5.2 and 4.8, respectively (Table 1), so the values predicted by the models at these exposure combinations should not be interpreted. Stepwise logistic and linear regressions were performed with the BMDPLR (Dixon et al. 1985) and SPSS (Nie et al. 1975; Neter et al. 1985) software packages, respectively. All statistical analyses were performed with the University of Wyoming's mainframe computers (Control Data Corp., Cyber 840).

Results and Discussion

Strain Comparisons

Survival and weight

Survival of both free-swimming strains (not confined in the PVC tubes) was reduced at similar levels of pH and aluminum (Fig. 1, Table 2). The predicted survival response surface was strongly dependent on combined aluminum and pH exposure (Fig. 1). Survival tended to be reduced at aluminum concentrations of 29 μg IAl/L at pH 5.2 and ⩾228 μg IAl/L at pH 4.4 and 4.8 for both strains, but exposure to low pH alone was neither observed (Table 2) or predicted (Fig. 1) to be toxic at the range tested. At toxic combinations of pH and aluminum, concurrent exposure to low calcium tended to reduce survival (Table 2), however, calcium was not a significant variable in the final regression model (Fig. 1).

Weight of both free-swimming strains was reduced at similar levels of pH and aluminum (Fig. 2, Table 2). The predicted weight response surface was strongly dependent on both aluminum and pH levels. Mean weight was consistently lower and weight change during the experiment was often negative with exposure to pH below 4.8 or aluminum concentrations ⩾34 μg IAl/L. Weight was not consistently affected with exposure to lower calcium concentrations at pH 4.4 (Table 2). Increasing calcium generally reduces either acid or aluminum toxicity (e.g. Ingersoll et al. 1985, 1990). The lack of a strong calcium effect on either weight or survival in the present study may be the result of the narrow range in calcium tested (1–2 mg Ca/L).

Exposure to elevated aluminum or acidity was observed to affect feeding behavior. Fish exposed to pH 6.5, 5.2, or 4.4 without aluminum addition consumed most of their food ration, whereas fish exposed to pH 4.4, 1 mg Ca/L and ⩾239 μg IAl/L seldom fed. Initially, food consumption was reduced at intermediate pH and aluminum exposure combinations. However, a resumption of feeding occurred during the study that was dependent on the severity of the exposure. Stressed fish often lost equilibrium, swam near the surface of the water, and secreted excessive amounts of mucus on the body surface and gills. The skin color of many of these fish darkened in either the anterior left or right quarter of the body suggesting possible neurological effects. Brook trout exposed to elevated levels of aluminum or acidity often exhibit reduced feeding (Menendez

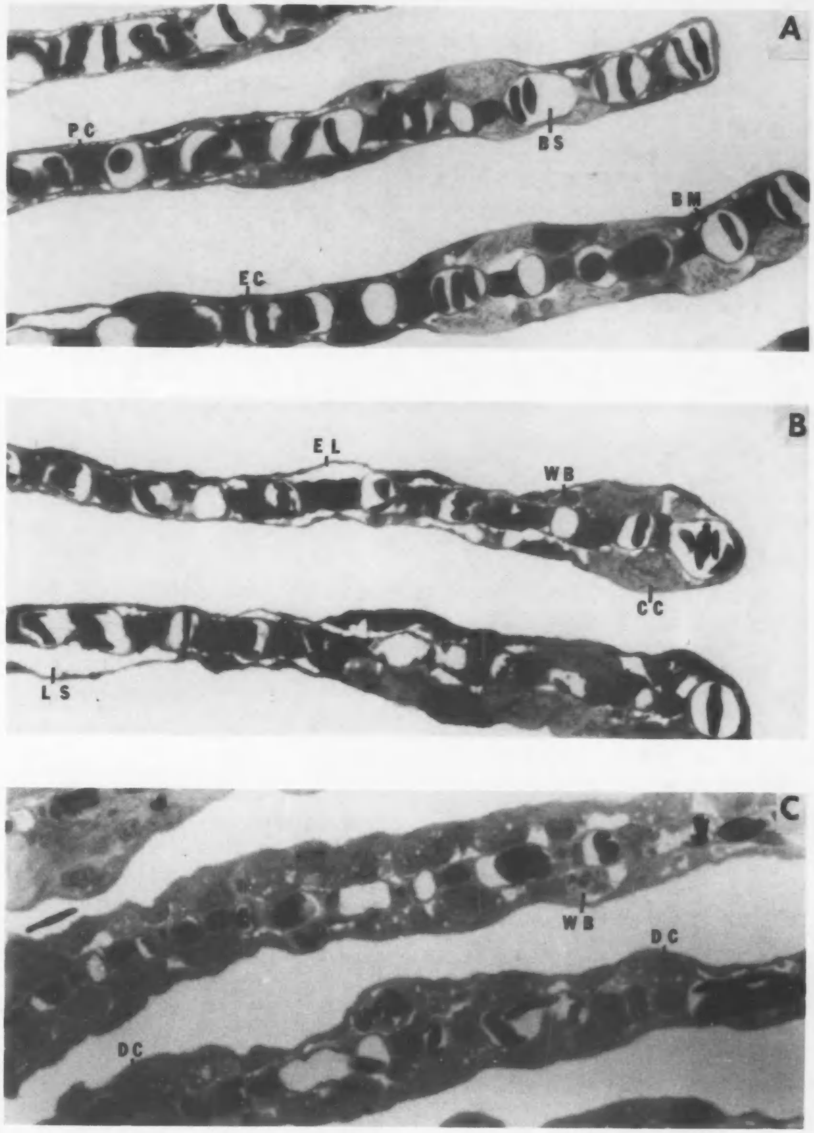


FIG. 3. Representative cross section of gill secondary lamellae (1000×) from NY-strain fish exposed to 2 mg Ca/L (nominal) and: (A) pH 6.5, 4 μg IAl/L, (B) pH 4.4, 4 μg IAl/L, and (C) pH 4.4, 228 μg IAl/L. PC = pillar cell, BS = blood space, BM = basement membrane, EC = epithelial cell, EL = epithelial lifting, WB = white blood cell, CC = chloride cell, LS = lymphatic space, DC = dense cell.

1976), loss of equilibrium (Swarts et al. 1978), increased activity (Johnson 1975), excessive mucus secretion (Dively et al. 1977), or changes in skin pigmentation (Schofield and Trojnar 1980). Many of these responses, including reduced growth (Kwain and Rose 1985; Tam et al. 1986), often subside after prolonged exposure, suggesting acclimation to stressful conditions (Wood et al. 1990a, b; Mount et al. 1990).

Our survival and weight data agree closely with those of Siddens et al. (1986) who report reductions in survival and weight of an unidentified strain of yearling brook trout after a 24-d exposure to elevated acid or aluminum. However, additional strains will need to be tested in order to more definitively determine intraspecific aluminum sensitivity for brook trout. Intraspecific differences in survival to low pH have been demonstrated for many fish species including brook trout (Johnson 1975; Robinson et al. 1976; Swarts et al. 1978), brown trout *Salmo trutta* (Gjedrem 1976; Edwards and Gjedrem 1979), Atlantic salmon *Salmo salar* (Schom 1986) and perch (Rahel

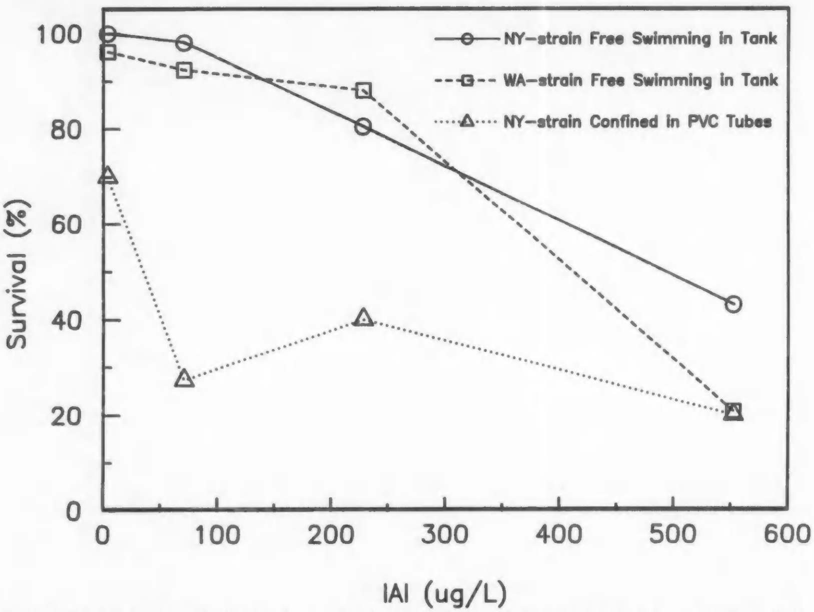


FIG. 4. Percent survival of New York-strain fish confined in PVC tubes and free-swimming New York- and Washington-strain fish at the designated aluminum concentrations. All fish were exposed to pH 4.4 and 2 mg Ca/L.

1983; Rask and Virtanen 1986), however, differences in size or age of fish tested confounded many of these strain comparisons (e.g. Johnson 1975; Edwards and Gjedrem 1979).

Gill histopathology

No qualitative differences in gill structure were observed between the New York- and Washington-strain fish with exposure to either low pH or elevated aluminum. The gills from fish exposed to pH 6.5 exhibited normal architecture (e.g. Tietge et al. 1988) for brook trout (Fig. 3). Gills sampled from fish exposed to low pH alone (pH 4.4, 4 μg IAl/L) showed some pathology with the most striking feature being a pronounced lifting of the outer epithelium away from the basement membrane and pillar cells. In addition, the chloride and epithelial cells of these fish appeared hypertrophic (increased size). The lifting and hypertrophy of the cells greatly increased the diffusion distance (water to blood distance). There was also evidence white blood cells influxed into the lymphatic spaces.

Gills sampled from fish exposed to low pH in combination with elevated aluminum (pH 4.4, 228 μg IAl/L) exhibited much more exaggerated effects relative to the gills from fish exposed to low pH alone. In addition, the gills from fish exposed to increased acid and aluminum exhibited both a hypertrophy and hyperplasia (increased number) of chloride cells as well as a hypertrophy of pavement epithelial cells. Many of the outer epithelial cells and chloride cells were quite vacuolated and appeared to be undergoing degeneration. Also evident in these gills was the presence of "dense cells" (Morrison 1979; Tietge et al. 1988) which ultrastructurally resemble chloride cells with the exception of fewer microvilli, and a disrupted tubular system. Aluminum stress at low pH may result in a proliferation of chloride cells with cytoplasmic inclusions believed to be high in aluminum (Karlsson–Norrgren et al. 1986; Tietge et al. 1988). Youson and Neville (1987) found that fish exposed to aluminum and low pH developed electron dense particles in the respiratory epithelium as well as the chloride cells. Electron microscopy and x-ray microanalysis revealed elevated aluminum in these membrane bound bodies.

Enclosure Comparison

Survival of New York-strain fish confined in the PVC tubes and exposed to pH 4.4 was generally reduced at aluminum concentrations that did not affect survival of either free-swimming New York- or Washington-strain fish (Fig. 4, Table 2). The confined fish were hyperactive and developed open sores on the ends of their snouts by the end of the experiment. These sores resulted from fish constantly battering into the nylon mesh at the ends of the PVC tubes. Ending weight of these confined fish was not dependent on pH, aluminum, or calcium exposure levels.

In situ enclosures have been used to assess the susceptibility of fish to acidic conditions in the field (e.g. Kennedy 1980; Johnson et al. 1987; Parkhurst et al. 1990). Investigators using caged fish often report greater toxicity at similar exposure levels compared to laboratory bioassays (e.g. Johnson et al. 1987). Caution must be taken when concluding from these in situ exposures that the laboratory predictions of mortality underestimate the magnitude of safe field levels. Fish confined in enclosures may be subjected to additional stress (e.g. high loading, limited food, handling) beyond the stress resulting from aluminum or acid exposure. Any enclosure introduces artificial physical, chemical, and biological conditions. Further research is needed to evaluate the influence of enclosure design on the response of confined organisms (Solemdal 1981; Walde and Davies 1984). In addition, determining the exposure concentration in in situ studies is difficult since water chemistry is not continuously monitored. Comparisons based on mean concentrations may underestimate toxicity because intermittent exposures are often more toxic than continuous exposures (Ingersoll and Winner 1982; Seim et al. 1984; Curtis et al. 1985; Siddens et al. 1986).

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References

Baker, J. P. 1984. Fishes, p. 5–74 to 5–139. *In* A. A. Altshuller and R. A. Linthurst [ed.] The acid deposition phenomenon and its effects; critical assessment review papers. Vol. II. Effects Sciences. EPA-600/8-83-016B. U.S. Environmental Protection Agency. Washington, DC.

Baker, J. P., and C. L. Schofield. 1980. Aluminum toxicity to fish as related to acid precipitation and Adirondack surface water quality, p. 292–293. *In* D. Drablos and A. Tollan [ed.] Ecological impact of acid precipitation: proceedings of an international conference, Sandefjord, Norway, March 11-14, 1980. SNSF-project, As-NLH, Norway.

1982. Aluminum toxicity to fish in acidic water. Water Air Soil Pollut. 18: 289–309.

1985. Acidification impacts on fish populations: a review. *In* D. D. Adams and W. P. Page [ed.] Acid Deposition. Plenum Press, New York, NY.

Bergman, H. L., J. S. Mattice, and D. J. A. Brown. 1988. Lake acidification and fisheries project: adult brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45: 1561–1562.

Bergman, H. L., and J S. Mattice. 1990. Lake acidification and fisheries project: brook trout (*Salvelinus fontinalis*) early life stages. Can. J. Fish. Aquat. Sci. 47: 1578–1579.

Curtis, L. R., W. K. Seim, and G. A. Chapman. 1985. Toxicity of fenvalerate to developing steelhead trout following continuous or intermittent exposure. J. Toxicol. Environ. Health 15: 445–457.

Dively, J. L., J. E. Mudge, W. H. Neff, and A. Anthony. 1977. Blood PO_2, PCO_2, and pH changes in brook trout (*Salvelinus fontinalis*) exposed to sublethal levels of acidity. Comp. Biochem. Physiol. 57A: 347–351.

Dixon, W. J., M. B. Brown, L. Engelman, J. W. Frane, M. A. Hill, R. I. Jennrich, and J. D. Toporek. 1985. BMDP statistical software. University of California Press, Berkeley, CA. 734 p.

Driscoll, C. T., J. P. Baker, J. J. Bisogni, and C. L. Schofield. 1980. Effect of aluminum speciation on fish in dilute acidified waters. Nature (Lond.) 284: 161–164.

Edwards, D. , and T. Gjedrem. 1979. Genetic variation in survival of brown trout eggs, fry and fingerlings in acidic water. SNSF project, FR 16/79, Oslo, Norway. 28 p.

Fivelstad, S., and H. Leivestad. 1984. Aluminum toxicity to Atlantic salmon (*Salmo salar* L.) and brown trout (*Salmo trutta* L.): mortality and physiological response, p. 69–77. Inst. Freshwater Res. Nat. Swed. Board of Fish. Rep. 61.

Gjedrem, T. 1976. Genetic tolerance of brown trout to acid water. SNSF project, Oslo, Norway. 11 p.

Haines, T. A., and J. P. Baker. 1986. Evidence of fish population responses to acidification in the eastern United States. Water Air Soil Pollut. 31: 605–629.

Hulsman, P. F., P. M. Powles, and J. M. Gunn. 1983. Mortality of walleye eggs and rainbow trout yolk-sac larvae in low-pH waters of the La Cloche mountain area, Ontario. Trans. Am. Fish. Soc. 112: 680–688.

Ingersoll, C. G. 1986. The effects of pH, aluminum, and calcium on survival and growth of brook trout (*Salvelinus fontinalis*) early life stages. PhD. thesis, University of Wyoming, Laramie, WY. 122 p.

Ingersoll, C. G., T. W. LaPoint, H. L. Bergman, and J. Breck. 1985. An early life stage brook trout (*Salvelinus fontinalis*) experiment to determine the effects of pH, calcium, and aluminum in low conductivity water, p. 42–48. *In* P. J. Rago and R. K. Schreiber [ed.]. Symp. Proc., 114th Ann. Meet., Am. Fish. Soc., Biol. Rep. 80(40.21): 106 p.

Ingersoll, C. G., D. R. Mount, D. D. Gulley, T. W. LaPoint, and H. L. Bergman. 1990. Effects of pH, aluminum, and calcium on survival and growth of eggs and fry of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: 1580–1592.

Ingersoll, C. G., and R. W. Winner. 1982. Effects on *Daphnia pulex* (DeGeer) of daily pulse exposures to copper or cadmium. Environ. Toxicol. Chem. 1: 321–327.

Johnson, D. W. 1975. Spawning behavior and strain tolerance of brook trout (*Salvelinus fontinalis*, Mitchill) in acidified water. MS. thesis, Cornell University, Ithaca, NY. 100 p.

Johnson, D. W., J. R. Colquhoun, F. M. Flack, and H. A. Simonin. 1987. *In Situ* toxicity tests of fishes in acid waters. Biogeochemistry. 3: 181–208.

Johnson, R. D. 1983. The use of quantitative histopathology in aquatic toxicology: Correlations between gill responses and routine toxicity measures in rainbow trout. PhD. thesis, University of Wyoming, Laramie, WY. p. 90.

Karlsson–Norrgren, L., I. Bjorklund, O. Ljungberg, and P. Runn. 1986. Acid water and aluminum exposure: experimentally induced gill lesions in brown trout, *Salmo trutta* L. J. Fish Dis. 9: 11–25.

Kennedy, L. A. 1980. Teratogenesis in lake trout (*Salvelinus namaycush*) in an experimentally acidified lake. Can. J. Fish. Aquat. Sci. 37: 2355–2358.

Kwain, W., and G. A. Rose. 1985. Growth of brook trout *Salvelinus fontinalis* subject to sudden reductions of pH during their early life history. Trans. Am. Fish. Soc. 114: 564–570.

LaZerte, B. 1984. Forms of aqueous aluminum in acidified catchments of southern Ontario: a methodological analysis. Can. J. Fish. Aquat. Sci. 41: 766–776.

Menendez, R. 1976. Chronic effects of reduced pH on brook trout (*Salvelinus fontinalis*). J. Fish. Res. Board Can. 33: 118–123.

Morrison, C. M. 1979. A dense cell in the epithelium of the gill lamellae of the brook trout, *Salvelinus fontinalis* (Mitchill). J. Fish Biol. 15: 601–605.

Mount, D. R., C. G. Ingersoll, D. D. Gulley, J. D. Fernandez, T. W. LaPoint, and H. L. Bergman. 1988. The effects of long-term exposure to acid, aluminum, and low calcium on adult brook trout (*Salvelinus fontinalis*): 1. Survival, growth, fecundity, and progeny survival. Can. J. Fish. Aquat. Sci. 45: 1623–1632.

Mount, D. R., M. J. Swanson, J. E. Breck, A. M. Farag, and H. L. Bergman. 1990. Responses of brook trout (*Salvelinus fontinalis*) fry to fluctuating acid, aluminum, and low calcium. Can. J. Fish. Aquat. Sci. 47: 1623–1630.

Neter, J., W. Wasserman, and M. H. Kutner. 1985. Applied linear statistical models. Richard D. Irwin, Inc. Homewood. IL: 1127 p.

Nie, N. H., C. H. Hull, J. G. Jenkins, K. Steinbrenner, and D. H. Bent. 1975. SPSS Statistical package for the social sciences. McGraw-Hill, New York, NY. 675 p.

Parkhurst, B. R. 1987. A comparison of laboratory and in situ bioassays for evaluating the toxicity of acidic water to brook trout. PhD. thesis, University of Wyoming, Laramie, WY. p. 101.

Parkhurst, B. R., H. L. Bergman, J. D. Fernandez, D. D. Gulley, J. R. Hockett, and D. A. Sanchez. 1990. A comparison of laboratory and *in situ* bioassays for evaluating the toxicity of acidic waters to brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 47: (In press)

Rahel, F. J. 1983. Population differences in acid tolerance between yellow perch, *Perca flavescens*, from naturally acidic and alkaline lakes. Can. J. Zool. 61: 147–152.

Rask, M., and E. Virtanen 1986. Responses of perch, *Perca fluviatilis* L., from an acidic and neutral lake to acidic water. Water Air Soil Pollut. 30: 537–543.

Robinson, G. D., W. A. Dunson, J. E. Wright, and G. E. Mamolito. 1976. Differences in low pH tolerance among strains of brook trout (*Salvelinus fontinalis*). J. Fish. Biol. 8: 5–17.

Rosseland, B. O., and O. K. Skogheim. 1984. A comparative study of salmonid fish species in acid aluminum-rich water. II. Physiological stress and mortality of one- and two-year-old fish, p. 186–194. Inst. Freshwater Res. Nat. Swed. Board Fish. Rep. 61.

Rosseland, B. O., O. K. Skogheim, and I. H. Sevaldrud. 1986. Acid deposition and effects in Nordic Europe. Damage to fish populations in Scandinavia continue to apace. Water Air Soil Pollut. 30: 65–74.

Schofield, C. L. 1982. Historical fisheries changes in the United States related to decreases in surface water pH, p. 57–67. *In* R. Johnson [ed.] Acid Rain/Fisheries. Northeastern Div. Am. Fish. Soc. Bethesda, MD.

Schofield, C. L., and J. R. Trojnar. 1980. Aluminum toxicity to brook trout (*Salvelinus fontinalis*) in acidified waters, p. 341–366. *In* T. Y. Toribara, M. W. Miller, and P. E. Morrow [ed.] Polluted Rain. Plenum Press, New York, NY.

Schom, C. B. 1986. Genetic, environmental, and maturational effects on Atlantic salmon (*Salmo salar*) survival in acute low pH trials. Can. J. Fish. Aquat. Sci. 43: 1547–1555.

Seim, W. K., L. R. Curtis, S. W. Glenn, and G. A. Chapman. 1984. Growth and survival of developing steelhead trout (*Salmo gairdneri*) continuously or intermittently exposed to copper. Can. J. Fish. Aquat. Sci. 41: 433–438.

Sevaldrud, I. H., and O. K. Skogheim. 1986. Changes in fish populations in southernmost Norway during the last decade. Water Air Soil Pollut. 30: 381–386.

Siddens, L., W. K. Seim, L. R. Curtis, and G. A. Chapman. 1986. Comparison of continuous and episodic exposure to acidic, aluminum-contaminated waters of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 43: 2036–2040.

Solemdal, P. 1981. Overview Enclosure Studies. Rapp. P.-V. Reun. Cons. Int. Explor. Mer. 178: 117–120.

Swarts, F. A., W. A. Dunson, and J. E. Wright. 1978. Genetic and environmental factors involved in increased resistance of brook trout to sulfuric acid solutions and mine acid polluted waters. Trans. Am. Fish. Soc. 107: 651–677.

Tam, W. H., P. D. Payson, and R. J. J. Roy. 1986. Retardation and recovery of growth in brook trout fry (*Salvelinus fontinalis*) exposed for various durations to acidified water. Can. J. Fish. Aquat. Sci. 43: 2048–2050.

Tietge, J.E., R. D. Johnson, and H. L. Bergman. 1988. Morphometric analysis of the secondary lamellae of brook trout (*Salvelinus fontinalis*) after long-term exposure to acid and aluminum. Can. J. Fish. Aquat. Sci. 45: 1643–1648.

Walde, S. J., and R. W. Davies. 1984. Invertebrate predation and lotic prey communities: evaluation of in situ enclosure/exclosure experiments. Ecology 65: 1206–1213.

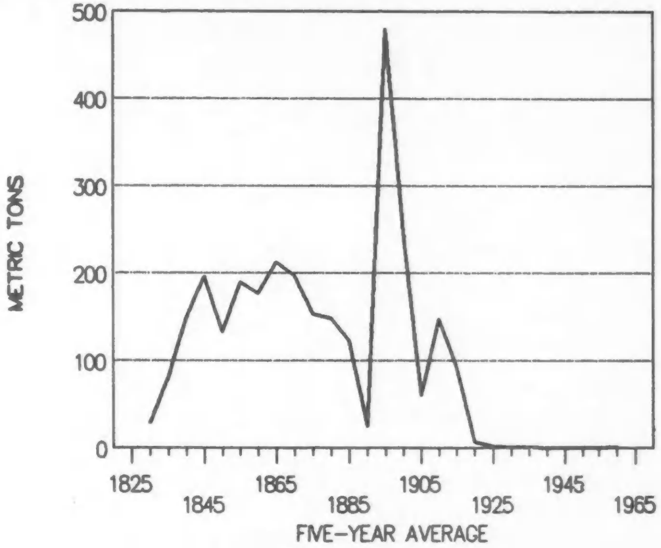
Wood, C. M., D. G. McDonald, C. G. Ingersoll, D. R. Mount, O. E. Johannsson, S. Landsburger, and H. L. Bergman. 1990a. Effects of water acidity, calcium, and aluminum on whole body ions of brook trout (*Salvelinus fontinalis*) continuously exposed from fertilization to swim-up: a study by instrumental neutron activation analysis. Can. J. Fish. Aquat. Sci. 47: 1593–1603.

1990. Whole body ions of brook trout (*Salvelinus fontinalis*) alevins: responses of yolk-sac and swim-up stages to water acidity, calcium, and aluminum, and recovery effects. Can. J. Fish. Aquat. Sci. 47: 1604–1615.

Youson, J. H., and C. M. Neville. 1987. Deposition of aluminum in the gill epithelium of rainbow trout (*Salmo gairdneri* Richardson) subjected to sublethal concentrations of the metal. Can. J. Zool. 65: 647–656.

CORRECTIONS TO RECENT PUBLICATIONS
CORRECTIONS SUR RÉCENTES PUBLICATIONS

VOLUME 45, No. 5 — note by T. Holzkamm and M. McCarthy: *Potential fishery for lake sturgeon (Acipenser fulvescens) as indicated by the returns of the Hudson's Bay Company Lac la Pluie district.* Page 923, Fig. 1: A calculation error regrettably led to significant underestimation of the sturgeon harvest between 1825 and 1885. The corrected figure is:



VOLUME 46, No. 8 — article by P. Bentzen, G. C. Brown, and W. C. Leggett: *Mitochondrial DNA polymorphism, population structure, and life history variation in American shad (Alosa sapidissima).* Page 1451, Table 4: The authors regret that a number of errors were missed in the upper half of this Table. The correct version is:

TABLE 4. Composite…(caption as published)…

Composite genotype	StJ's*	Alta*	Coop*	Wacc*	Pam*	York	Dela	Huds	Conn	Anna	StJ	Mir	StLaw	Rich	Σ
S,L AAAA	5			2	1	2	1								11
S,L AABA	2			2											4
S,L ABAA	1	2								1					4
S,L BBAA		1	1												2
S,L AAAB							1			1					2
S AAAA	6	4	9	7	6	8	10	6	4	2	5	5	5	2	79
S AABA	2	2		1	1	2		1		2	1	7	14	1	34
S ABAA		5	2	1		3				1	3	3	3		21
S BBAA		2		1	5	2	4	3	2		4				23
S AACA		1													1
S AAFA			1												1
S A,BBAA			1			2	1			1					5
S AAAB				3	3	2		7	7	11	1				34
S A,BAAA					1		1			1					3
S BABA					1										1
S ABAB					1		1								2
S ACAA						1									1
S BAAA							1					3	1		5
S AACB							1								1
S BAAB							1								1
S AAEA							1								1
S AADA								1							1

TABLE 4. (*Continued*)

Composite genotype	StJ's*	Alta*	Coop*	Wacc*	Pam*	York	Dela	Huds	Conn	Anna	StJ	Mir	StLaw	Rich	Σ
S AADB								1							1
S A,BAEA								1							1
S AAA-[1]														2	2
S AAB-[1]														2	2
Σ	16	17	14	17	19	22	23	20	13	20	14	18	23	7	243

[1] *Eco*RV did not produce clear digestion patterns with these samples.

VOLUME 47, No. 4 — article by R. W. Sterner: *Lake morphometry and light in the surface layer*.

Page 689: Half way down the left-hand column, the printer regrettably omitted Equation (3) following "...Reynolds 1984; Rott 1986):". It should have read:

$$(3) \quad K \approx \Phi / z_{sd}.$$

VOLUME 47, No. 5 — article by L. A. Giguère and R. L. Dunbrack: *Thin layer interference may reduce visibility of transparent phantom midge larvae (Chaoborus trivittatus) to predators*.

Page 1043: The following footnote was regrettably omitted from the first author's name: "Order of authorship determined by the flip of a coin. Authors contributed equally."

Parkhurst, B. R., H. L. Bergman, J. Fernandez, D. D. Gulley, J. R. Hockett, and D. A. Sanchez. Inorganic monomeric aluminum and pH as predictors of acidic water toxicity to brook trout (*Salvelinus fontinalis*) **1631–1640**

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Corrections to recent publications/Corrections sur récentes publications **1649–1650**

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